

REMARKS

Upon entry of the present Amendment, Claims 68 and 108-145 will be pending in this application. The changes made to the claims by the current amendment, including deletions and additions, are shown herein with deletions designated with a ~~striketrough~~ and additions underlined. As agreed at the interview, the amended claims do not include new matter.

Claim 68 has been amended as discussed at the interview. All of the dependent claims have been amended to refer to "the construct" rather than "a construct" as requested by the Examiner in the objection to the claims. The dependency of claims 123 and 124 has been revised in view of the amendment of Claim 68 and the cancellation of Claim 107. In addition, Claims 138, 144 and 145 have been amended to be consistent with the amendment of Claim 68.

Rejection Pursuant to 35 U.S.C. §101

The rejection under 35 U.S.C. §101 applied only to Claim 102, which has been canceled herein. Accordingly, this rejection is moot.

Rejection of Claims Pursuant to 35 U.S.C. §112, Second Paragraph

The word "polypeptide" has been restored to Claim 68, so as to overcome the rejection under 35 U.S.C. §112, Second Paragraph.

Rejections of Claims Pursuant to 35 U.S.C. §112, first paragraph

As discussed above in the Summary of the Interview, the Examiner has agreed to remove the written description rejection of Claim 68 and the claims dependent thereon in view of the arguments presented at the interview.

As also discussed above, the specification discloses more than merely "a species of RNA elements that destabilize the stability of a transcript that are AU rich elements or U rich elements as disclosed in SEQ ID NO:s 1-23." Specifically, DST elements are disclosed in Paragraph [0342] of the published specification. As shown in the specification, these elements can be as low as 40% AU. Additionally, Histone elements are disclosed at Paragraph [0363]. The specification shows that these elements can actually be GC-rich, at as low as 16% AU. Moreover, the coding region instability element *c-fos* is not AU rich. The attached reference by Shyu et al., *Genes and Development*, 3:60-72 (1989) states that "the *c-fos* coding sequence contains neither a long AU-rich segment nor the pentanucleotide AUUUA found in all known

AREs.” Additionally, Chen et al., *Trends Biochem. Sci.*, 20:465-470 (1995), also attached, define the *c-fos* coding region instability element as purine-rich (i.e. AG-rich).

In view of the agreement at the interview, and the scientific support herein presented, withdrawal of the rejection under 35 U.S.C. §112, first paragraph is respectfully requested.

Rejection of Claims Over Prior Art

As discussed above in the Summary of the Interview, the Examiner has agreed to remove the prior art rejections of Claim 68 and the claims dependent thereon in view of the arguments presented at the interview.

As also discussed above, Zhao et al. discloses the use of the SV40 polyadenylation signal, and the Eureka reference discloses that this element might lead to RNA destabilization in prokaryotes. However, the claims have been amended to recite an “RNA destabilizing element that reduces the stability of a transcript encoded by the polynucleotide in a eukaryotic cell.” The Chen et al. (1995) reference discussed above shows that removal, not presence, of poly-A tails causes destabilization of RNA in eukaryotes. In addition, the attached copy of Kushner, *Life*, 56:585-594 (2004) provides a review of the different effects of poly-A tails in eukaryotic and prokaryotic cells, and concludes that the presence of poly-A tails in eukaryotes, such as yeast, protects transcripts from degradation. Two different publications authored by Bernstein and others in 1989 (*Trends Biochem. Sci.*, 14:373-377 and *Mol. Cell. Biol.*, 9:659-670) are also attached that further support this conclusion by showing that stable mRNA becomes unstable when its poly-A tail is removed. Thus, the presence of poly-A actually protects transcripts from degradation in eukaryotes, the opposite of the claimed invention.

As agreed at the interview, the other cited references all disclose RNA-destabilization in prokaryotes, which, as discussed above, is quite distinct from the recited elements that reduce stability in eukaryotes. Accordingly, removal of the rejections over the prior art of record is respectfully requested.

Conclusion

In view of the foregoing amendments and remarks, the application is presented as in condition for allowance. Should the Examiner identify any remaining issues which might prevent the prompt issuance of a Notice of Allowance, the Examiner agreed at the interview that she would contact the undersigned attorney at the telephone number appearing below prior to issuing a written Office Action.

Appl. No. : 10/658,093
Filed : September 9, 2003

No fees are believed due. However, please charge any necessary fees, including any fees for extensions of time, to Deposit Account No. 11-1410.

Respectfully submitted,

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The *c-fos* transcript is targeted for rapid decay by two distinct mRNA degradation pathways

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Rapid degradation of *c-fos* proto-oncogene mRNA is crucial for transient *c-fos* gene expression. Experiments were performed to investigate the cellular mechanisms responsible for the extremely short half-life of human *c-fos* mRNA in growth-factor-stimulated fibroblasts. These experiments demonstrate the existence of two distinct cellular pathways for rapid *c-fos* mRNA degradation. Each of these pathways recognizes a different, functionally independent instability determinant within the *c-fos* transcript. One instability determinant, which is located within the *c-fos* 3'-untranslated region, is a 75-nucleotide AU-rich segment. Insertion of this element into β -globin mRNA markedly reduces the half-life of that normally long-lived message. Nevertheless, specific deletion of the AU-rich element from *c-fos* mRNA has little effect on the transcript's cytoplasmic half-life due to the presence of the other *c-fos* instability determinant, which is located in the protein-coding segment of the *c-fos* message. Examination of mRNA decay in cells treated with transcription inhibitors indicates that one *c-fos* mRNA degradation pathway is dependent on RNA synthesis, whereas the other is not.

[Key Words: mRNA degradation; *c-fos*; AU-rich element; proto-oncogene]

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An important mechanism for regulating gene expression in all organisms is control of the cytoplasmic concentration of individual gene transcripts. mRNA levels often change dramatically during cell growth and differentiation as a result of changes in transcription, RNA processing, and mRNA decay. Although a great deal has been learned in recent years about the regulation of mRNA synthesis, much less is known about the cellular mechanisms that control mRNA degradation. Nevertheless, a number of studies have revealed that bacterial and mammalian gene expression can be modulated through changes in mRNA stability (for reviews, see Belasco and Higgins 1988; Brawerman 1987).

In mammalian cells, a class of 10–50 growth-factor-inducible genes has been identified whose transcripts appear transiently in the cytoplasm immediately after stimulation with growth factors (Greenberg and Ziff 1984; Lau and Nathans 1985; Lim et al. 1987; Almendral et al. 1988). The transient expression of these genes is a consequence not only of the brevity of their transcription but also of their transcripts' very short lifetime in the cytoplasm. The lability of this class of mRNA, which decays with a half-life of <30 min, distinguishes it from most mammalian messages, which typically are much more stable, with half-lives ranging from hours to days (Krowczynska et al. 1985). As yet, little is understood about the mechanisms by which these growth-factor-inducible transcripts are degraded.

The best characterized of the growth-factor-inducible messages is the transcript of the *c-fos* proto-oncogene.

The *c-fos* gene encodes a nuclear protein that forms a complex with the mammalian transcription factor AP-1 and is believed to regulate the transcription of a diverse set of genes important for cell growth and differentiation (Chiu et al. 1988; Rauscher et al. 1988; Sassone-Corsi et al. 1988). Expression of the *c-fos* gene is induced rapidly and transiently in a wide range of mammalian cell types by growth factors, phorbol esters, neurotransmitters, and membrane-depolarizing agents (Greenberg and Ziff 1984; Curran and Morgan 1985, 1986; Greenberg et al. 1985, 1986b). Induction of *c-fos* occurs at the transcriptional level and is independent of new protein synthesis (Cochran et al. 1984; Greenberg et al. 1986a). Minutes after its activation, transcription of *c-fos* ceases (Greenberg et al. 1985, 1986b). The newly synthesized *c-fos* mRNA is transported to the cytoplasm, where it is translated for only a brief period of time before being degraded rapidly (Kruijer et al. 1984; Muller et al. 1984). This transient accumulation of *c-fos* mRNA appears to be critical to normal cellular function because mutations that result in its deregulated expression can lead to oncogenic transformation (Miller et al. 1984; Meijlink et al. 1985; Jenuwein and Muller 1987).

There has been considerable interest in understanding the molecular basis for the rapid turnover of *c-fos* mRNA. Initial experiments showed that substitution of heterologous RNA for the 3' half of the *c-fos* transcript can have a stabilizing effect (Treisman 1985). The *c-fos* 3'-untranslated region (UTR) contains an AU-rich element (ARE, Fig. 1) that resembles an RNA segment pre-

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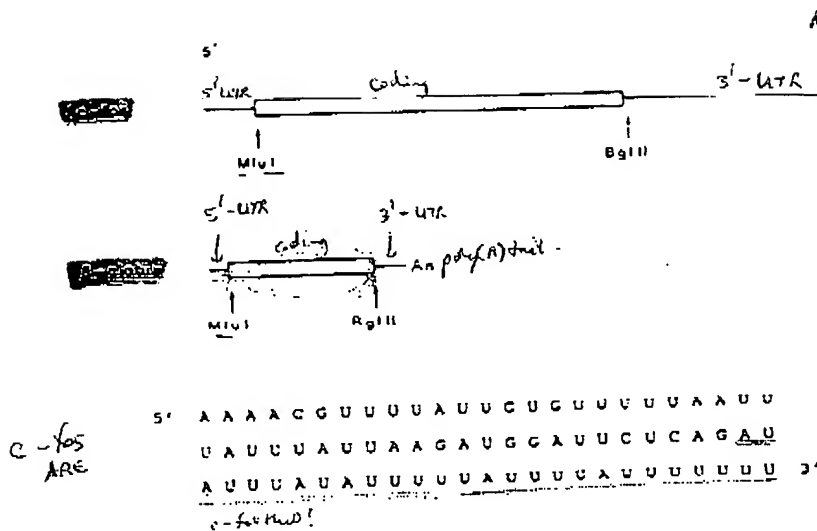


Figure 1. The *c-fos* and β -globin transcripts. (Top) Diagrams of *c-fos* and β -globin mRNA. (Thin lines) 5' and 3' UTRs; (open rectangles) protein-coding regions; (solid rectangle) ARE. (A_n) 3' poly(A) tail. Arrows mark sites that correspond to DNA restriction sites used in the construction of *c-fos*/ β -globin gene fusions. (*Mlu*I) A restriction site created in the *c-fos* and β -globin genes by three point mutations near the boundary between the 5' UTR and the protein-coding region; (*Bgl*III) a restriction site that is located immediately downstream of the rabbit β -globin translation termination codon and that was introduced into the *c-fos* gene by linker insertion at an *Nae*I site near the beginning of the *c-fos* 3' UTR. (Bottom) Sequence of the 75-nucleotide ARE of human *c-fos* mRNA.

viously implicated in the lability of granulocyte-monocyte colony-stimulating factor (GM-CSF) mRNA (van Straaten et al. 1983; Shaw and Kamen 1986). Large deletions in the *c-fos* 3' UTR that remove the ARE appear to retard *c-fos* mRNA degradation in actively growing fibroblasts when the *c-fos* gene is constitutively transcribed from a heterologous promoter and decay is monitored in the presence of the transcription inhibitor actinomycin D (Fort et al. 1987; Rahmsdorf et al. 1987). Nevertheless, the role of the ARE in determining the half-life of *c-fos* mRNA under normal conditions of transient gene expression in growth-factor-stimulated cells has not been established.

Here, we report experiments that reveal the existence of two distinct cellular pathways for rapid *c-fos* mRNA degradation. Each of these pathways recognizes a different *c-fos* instability determinant, either of which is sufficient to render the *c-fos* message highly labile. One of the instability determinants is the ARE in the 3' UTR; the other is a novel element within the *c-fos* protein-coding region. Remarkably, deletion of the ARE alone has little effect on *c-fos* mRNA instability in growth-factor-stimulated fibroblasts. We hypothesize that mammalian cells have evolved multiple *c-fos* mRNA decay mechanisms to ensure that this transcript is degraded rapidly within minutes after it is synthesized.

Results

Synthesis and decay of human *c-fos* mRNA in NIH-3T3 cells

To identify determinants responsible for the marked lability of *c-fos* mRNA, a transient-induction assay was employed to monitor the decay of human *c-fos* mRNA in mouse fibroblasts after growth-factor stimulation. A wild-type genomic clone of the human *c-fos* gene (pF711) was introduced into mouse NIH-3T3 cells by transient transfection. After serum starvation for 25–30 hr, *c-fos* transcription was induced transiently by stimulation with fetal calf serum, and total cytoplasmic RNA

was isolated at time intervals. The relative concentrations of human *c-fos* mRNA, mouse *c-fos* mRNA, and α -globin mRNA (a cotransfected internal standard) at various times after serum stimulation were then determined by RNase-protection analysis of equal amounts of each RNA sample, using a mixture of uniformly radiolabeled RNA probes (Fig. 2, top).

Transcription of both the transfected human *c-fos* gene and the endogenous mouse *c-fos* gene was stimulated transiently by serum addition, and these two messages decayed with similar kinetics (half-lives of 15 ± 3 min and 16 ± 1 min, respectively; Fig. 2, top) following the spontaneous cessation of *c-fos* transcription that occurred within 1 hr after serum induction (Greenberg and Ziff 1984; Greenberg et al. 1985). Efficient degradation of the human message required transfection with no more than 5 μ g of pF711 per plate of 2×10^6 cells; transfection with 20 μ g of pF711 per plate increased the apparent half-life of this transcript to 90–100 min, possibly by saturating the cellular machinery involved in *c-fos* mRNA degradation. α -Globin mRNA, which was synthesized constitutively under the control of an SV40 enhancer and did not decay detectably over the time course of these experiments, served as an internal control that allowed correction for small variations in transfection efficiency and sample handling.

These findings show that the decay of human *c-fos* mRNA and endogenous mouse *c-fos* mRNA can be studied in parallel in transiently transfected NIH-3T3 cells under conditions that are physiologically relevant to normal *c-fos* gene expression and without the use of drugs to inhibit transcription.

The *c-fos* 3' UTR is not required for *c-fos* mRNA instability

Experiments in other laboratories with *c-fos* genes transcribed from heterologous promoters have implicated an AU-rich segment of the *c-fos* 3' UTR (Fig. 1) in causing *c-fos* mRNA instability (Fort et al. 1987; Rahmsdorf et al. 1987). To test whether the ARE is necessary for the short cytoplasmic half-life of the *c-fos* transcript after

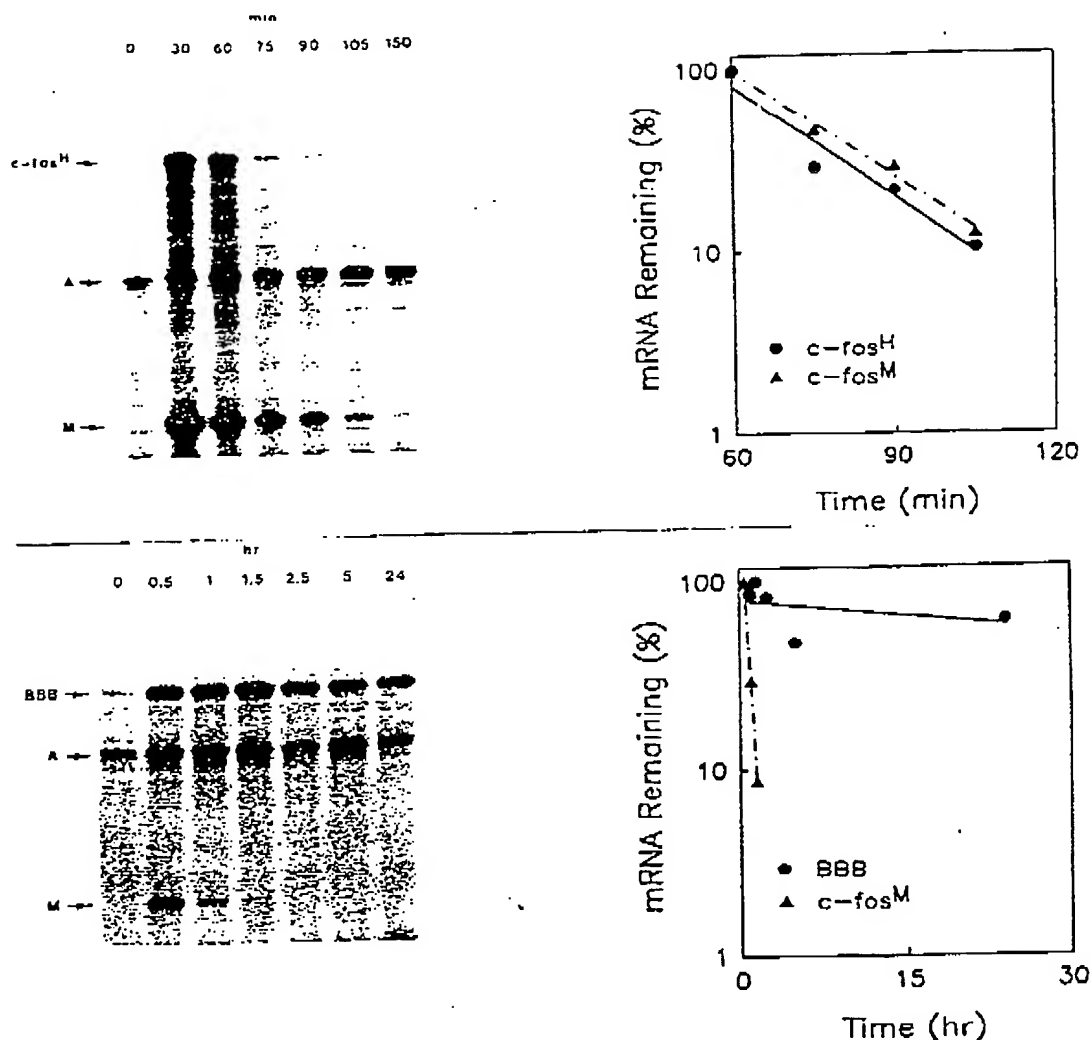


Figure 2. Induction and decay of human *c-fos* mRNA and rabbit β -globin mRNA. NIH-3T3 cells were transiently transfected with pSVa1 and either pF711 (top) or pBBB (bottom). Total cytoplasmic RNA was isolated at time intervals after serum stimulation and analyzed by RNase protection and gel electrophoresis. (c-fos^H) pSP6fos-derived probe fragment protected by human *c-fos* mRNA; (BBB) pT7BG-derived probe fragment protected by BBB mRNA; (M) pSP6fos-derived probe fragment protected by mouse *c-fos* mRNA; (A) pSP6a133-derived probe fragment protected by α -globin mRNA. Times correspond to minutes (top) or hours (bottom) after serum stimulation. Beside each autoradiogram is a semilogarithmic plot of the decay of human *c-fos* (c-fos^H) or rabbit β -globin (BBB) mRNA (solid line) and mouse *c-fos* mRNA (c-fos^M, dashed/dotted line).

growth-factor stimulation, a deletion was introduced into the human *c-fos* gene that precisely removed this 75-bp element (Table 1). The cytoplasmic half-life of the transcript of the resulting *c-fos* allele (FΔ1) was then determined after serum induction of transiently transfected NIH-3T3 fibroblasts. Surprisingly, the half-life of this message was found to be only 20 ± 3 min (Fig. 3, top), a value very similar to that measured for wild-type *c-fos* mRNA (15 ± 3 min). This finding demonstrates that the *c-fos* ARE is not required for the instability of *c-fos* mRNA in serum-stimulated fibroblasts.

To test for other structures in the long *c-fos* 3'-UTR that might contribute to *c-fos* mRNA lability, a nested

set of deletions was created within this region. These deletions removed the ARE in the *c-fos* 3'-UTR and extended unidirectionally for various distances (56–574 bp) upstream from it (FΔ2–FΔ6, Table 1). The cytoplasmic half-lives of the transcripts of the resulting *c-fos* alleles were measured as before, and all were found to decay at about the same rate as wild-type *c-fos* mRNA (Fig. 3, bottom; Table 1). The transcript of an additional *c-fos* deletion mutant (FΔ7; Table 1) that lacked a 201-nucleotide segment immediately upstream of the polyadenylation signal (AAUAAA), including the ARE, also decayed with a short half-life of 15 ± 1 min. Taken together, these findings demonstrate that no single ele-

Table 1. Half-lives of *c-fos* transcripts with deletions in the 3'-UTR

Deletion mutant	Deletion end points (kbp) ^a	Half-life (min)
Wild-type	—	15 = 3
FΔ1	1.92–2.00	20 = 3
FΔ2	1.87–2.00	18 = 4
FΔ3	1.67–2.00	15 = 4
FΔ4	1.60–2.00	20 = 2
FΔ5	1.51–2.00	21 = 1
FΔ6	1.35–2.00	18 = 1
FΔ7	1.87–2.07	15 = 1

^aEnd points are measured in kilobases from the 5' terminus of human *c-fos* mRNA. In the wild-type *c-fos* message, the 5'-UTR extends from nucleotide 1 to 155, the protein-coding region from 156 to 1295, and the 3'-UTR from 1296 to 2104. The *c-fos* ARE is located between positions 1920 and 1994.

ment >30–50 nucleotides from either end of the 809 nucleotide 3'-UTR of *c-fos* mRNA, including the AU-rich element, is necessary for *c-fos* mRNA instability.

The 3'-UTR deletions described above left intact the RNA segment between the polyadenylation signal and the site of poly(A) addition. To test whether this segment contains an instability determinant, we replaced all but the first 27 nucleotides of the *c-fos* 3'-UTR with the 3'-UTR of the rabbit β -globin message. As shown below, rabbit β -globin mRNA is very stable in serum-stimulated fibroblasts. The resulting hybrid transcript, which should be properly polyadenylated at the β -globin poly(A) site, was designated FFB. This name indicates that the 5'-UTR and the coding region were derived from the human *c-fos* message, whereas the 3'-UTR was derived from rabbit β -globin mRNA. In this three-letter nomenclature for gene fusions transcribed under the control of the *c-fos* promoter, the first letter refers to the origin of the 5'-UTR (F if from human *c-fos* and B if from rabbit β -globin), the second letter indicates the source of the coding region, and the third letter refers to the source of the 3'-UTR. When the FFB gene was introduced into NIH-3T3 cells by transient transfection, cytoplasmic half-life of its transcript after serum induction was found to be only 21 = 1 min (Fig. 4, top), about the same as that of wild-type *c-fos* mRNA. The instability of this *c-fos* mRNA variant, which lacked nearly the entire *c-fos* 3'-UTR, confirmed that the *c-fos* 3'-UTR is not necessary for *c-fos* mRNA instability.

Insertion of the c-fos ARE can destabilize an otherwise long-lived message

The lability of *c-fos* mRNA lacking the 3'-UTR could be explained in either of two ways. One possibility is that the *c-fos* 3'-UTR does not contain a signal for rapid mRNA degradation. Alternatively, there may be a determinant of mRNA instability in the 3'-UTR (e.g., the ARE) that is dispensable because of the presence of one or more additional instability determinants located up-

stream of the 3'-UTR. This additional element would ensure rapid decay of *c-fos* mRNA even when the 3'-UTR is absent. To ascertain whether the *c-fos* 3'-UTR, coding region, and 5'-UTR contain determinants of mRNA instability, we decided to substitute each of these *c-fos* mRNA segments for the corresponding segment of the stable rabbit β -globin transcript and to measure the decay rate of the resulting hybrid messages (BBF, BFB, and FBB).

As a first step, a *c-fos*/ β -globin gene fusion was constructed in which the β -globin promoter had been replaced precisely with the serum-inducible *c-fos* promoter. This gene fusion was designated BBB to indicate that it encoded a wild-type rabbit β -globin transcript whose 5' end mapped to the normal cap site. Induction and decay of β -globin mRNA transcribed from the *c-fos* promoter was then monitored in transfected NIH-3T3 cells to learn whether fusion of the *c-fos* promoter to a heterologous gene would allow transient transcription of that gene in response to serum stimulation. As in the case of the *c-fos* gene, BBB transcription was induced rapidly by addition of serum (Fig. 2, bottom). However, unlike *c-fos* mRNA, which disappears rapidly from the cytoplasm after cessation of transcription (Fig. 2, top), the cytoplasmic concentration of β -globin mRNA transcribed from the *c-fos* promoter reached a plateau within 1 hr after serum stimulation and did not decline significantly for at least 24 hr thereafter. These findings indicate that the *c-fos* promoter can direct transient synthesis of β -globin mRNA in response to serum stimulation and that the half-life of β -globin mRNA in serum-stimulated cells is very long (>24 hr).

To test whether the *c-fos* 3'-UTR contains a determinant of mRNA instability, we next analyzed the decay of a hybrid message (BBF) in which the 3'-UTR of β -globin mRNA was replaced with the corresponding segment of the *c-fos* transcript. As in the case of the BBB gene, transcription of the BBF gene was directed by the *c-fos* promoter. The cytoplasmic half-life of the chimeric BBF transcript was only 28 \pm 2 min (Fig. 4, bottom), a value almost as short as that of *c-fos* mRNA itself and <2% as long as that of wild-type β -globin (BBB) mRNA. Thus, although deletion of the *c-fos* 3'-UTR does not stabilize *c-fos* mRNA, this mRNA segment is able to confer a high degree of instability upon the otherwise long-lived β -globin message.

Further analysis showed that the functional element primarily responsible for the instability of BBF mRNA was the AU-rich segment within the *c-fos* 3'-UTR. Precise deletion of the 75-nucleotide *c-fos* ARE from BBF stabilized the message considerably (BBF Δ ARE; Fig. 5, top). Moreover, simple insertion of the *c-fos* ARE into the 3'-UTR of an otherwise intact β -globin message (to generate BBB + ARE) was highly destabilizing; this insertion reduced the half-life of β -globin mRNA from >24 hr to only 37 = 6 min (Fig. 5, bottom).

Taken together, these findings indicate that the *c-fos* ARE functions as an mRNA-destabilizing element. However, because deletion of the ARE or the entire 3'-UTR fails to stabilize *c-fos* mRNA, it appears that the *c-fos* message contains one or more additional deter-

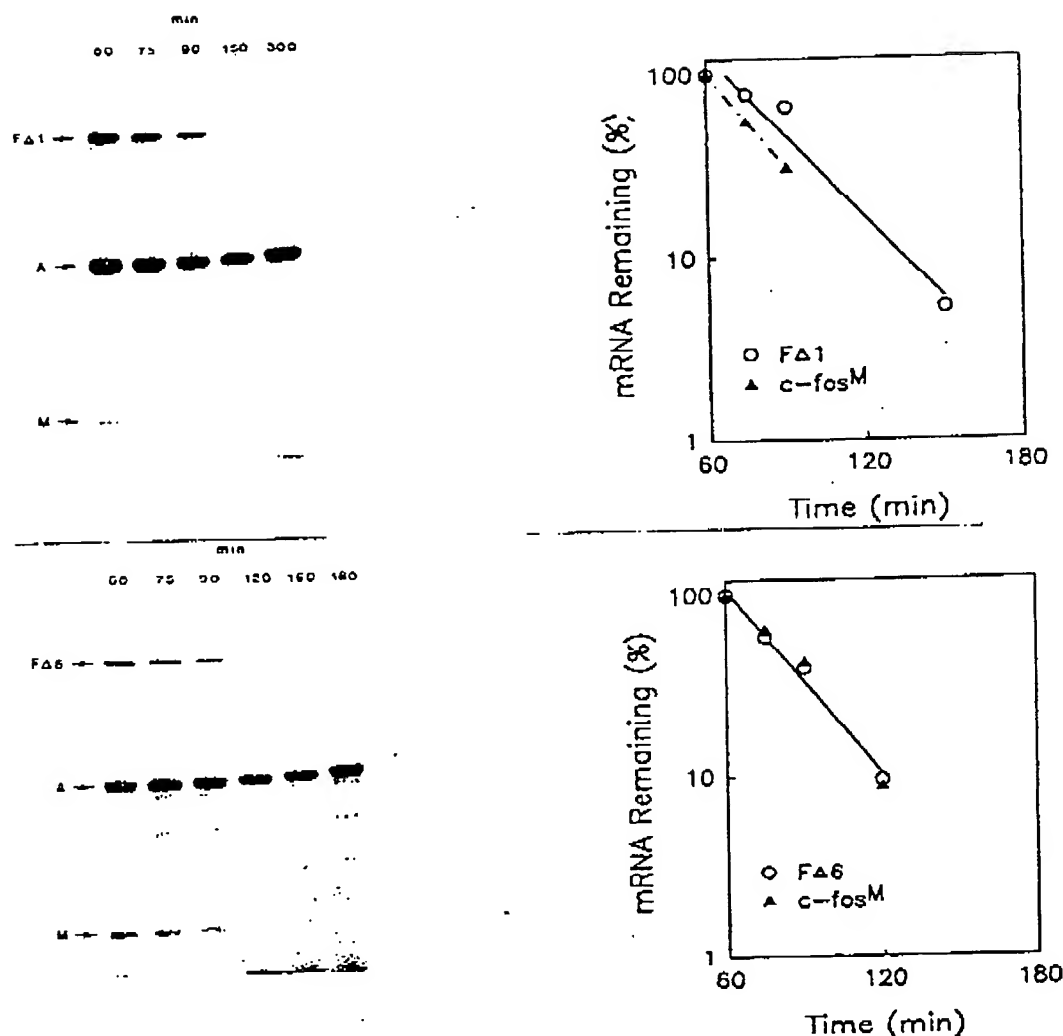


Figure 3. Decay of human *c-fos* mRNA with deletions in the 3'-UTR. NIH-3T3 cells were transiently transfected with pSVa1 and either pFA1 (top) or pFA6 (bottom). Total cytoplasmic RNA was isolated at time intervals after serum stimulation and analyzed by RNase protection and gel electrophoresis. (FA1) pSP6fos-derived probe fragment protected by FA1 mRNA; (FA6) pSP6fos-derived probe fragment protected by FA6 mRNA; (M) pSP6fos-derived probe fragment protected by mouse *c-fos* mRNA; (A) pSP6a133-derived probe fragment protected by α -globin mRNA. Times correspond to minutes after serum stimulation. Beside each autoradiogram is a semi-logarithmic plot of the decay of FA1 or FA6 mRNA (solid line) and mouse *c-fos* mRNA (*c-fos*^M, dashed/dotted line)

minants of instability in the 5'-UTR or in the coding region.

The c-fos coding region contains a determinant of mRNA instability

To map the location of the additional determinant(s) of *c-fos* mRNA instability, gene fusions were constructed in which the β -globin coding region or 5'-UTR was replaced with the corresponding segment of the *c-fos* gene (BFB and FBB). The construction of these gene fusions was facilitated by the introduction of point mutations into plasmid clones of the *c-fos* and β -globin genes that

resulted in the creation of a unique *Mlu*I restriction site 5 bp downstream of the ATG translation initiation codon of each gene. These point mutations per se did not affect the half-life of the β -globin message (A.-B. Shyu, unpubl.). Transcription of each gene fusion was directed by the *c-fos* promoter and could be transiently induced by serum stimulation.

Replacement of the β -globin coding region with that of *c-fos* (BFB) had a dramatic destabilizing effect: BFB mRNA decayed with a half-life of only 17 ± 2 min (Fig. 6, top). Thus, like the *c-fos* 3'-UTR, the *c-fos*-coding region contains a determinant of mRNA instability that alone is sufficient to destabilize an otherwise long-lived

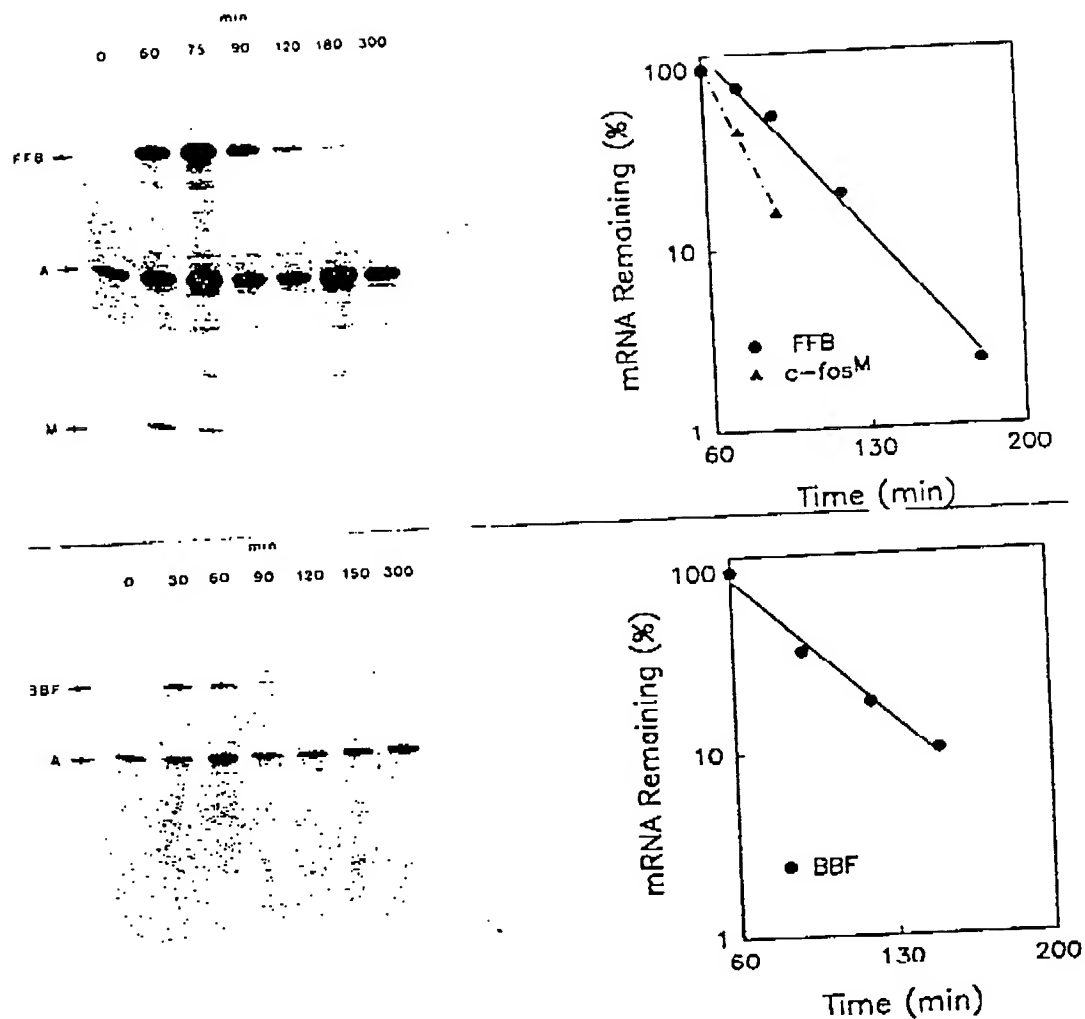


Figure 4. Decay of FFB and BBF mRNA. NIH-3T3 cells were transiently transfected with pSV α 1 and either pFFB (top) or pBBF (bottom). Total cytoplasmic RNA was isolated at time intervals after serum stimulation and analyzed by RNase protection and gel electrophoresis. (FFB) pSP6 α -derived probe fragment protected by FFB mRNA; (BBF) pT7BC-derived probe fragment protected by BBF mRNA; (M) pSP6 α -derived probe fragment protected by mouse *c-fos* mRNA; (A) pSP6 α 133-derived probe fragment protected by globin mRNA. Times correspond to minutes after serum stimulation. Beside each autoradiogram is a semilogarithmic plot of the decay of FFB or BBF mRNA (solid line) and mouse *c-fos* mRNA (*c-fos*^M, dashed/dotted line).

message. The instability determinant within the coding region apparently is more effective at destabilizing mRNA than even the ARE, inasmuch as the BBF transcript decays about twice as fast as BBB + ARE mRNA. In contrast to the 3'-UTR and coding region, substitution of the *c-fos* 5'-UTR for that of β -globin (BBF) hardly affected the cytoplasmic stability of the β -globin transcript, the half-life of FFB mRNA being >10 hr (Fig. 6, bottom).

Each c-fos instability determinant is the target of a distinct degradation pathway

To begin to characterize degradation mediated by each of the determinants of *c-fos* mRNA instability, we ex-

amined the effect of actinomycin D on message decay. Actinomycin D is a transcription inhibitor that acts by intercalating into nucleic acids. Interestingly, we discovered that actinomycin D differentially affects the function of the instability determinants located in the 3'-UTR and the protein-coding region. Treatment of serum-stimulated cells with actinomycin D gradually inhibited degradation of BBF mRNA. Within 0.5 hr after addition of actinomycin D, this message decayed slowly with a half-life of ~ 110 min, and it ceased to decay altogether within 3 hr (Fig. 7, top). In contrast, actinomycin D treatment had only a small effect on the decay of FFB mRNA, whose half-life was 28 ± 2 min in the presence of this drug and did not change with time (Fig. 7, top); nor did actinomycin D significantly affect the half-life of

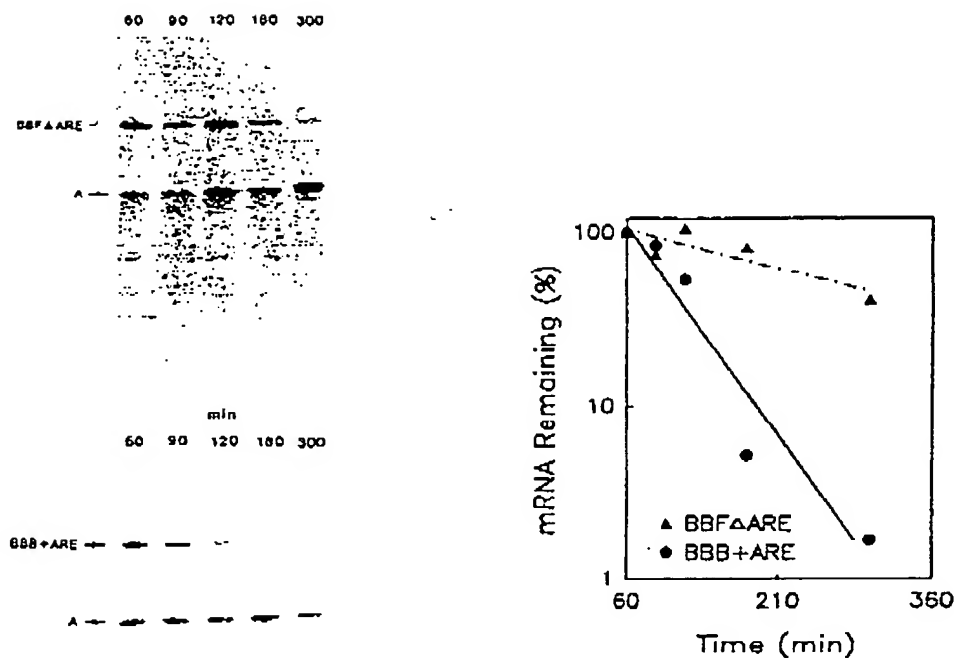


Figure 5. Decay of BBF Δ ARE and BBB + ARE mRNA. NIH-3T3 cells were transiently transfected with pSV α 1 and either pBBF Δ ARE (top) or pBBB + ARE (bottom). Total cytoplasmic RNA was isolated at time intervals after serum stimulation and analyzed by RNase protection and gel electrophoresis. (BBF Δ ARE) pT7BG-derived probe fragment protected by BBF Δ ARE mRNA; (BBB + ARE) pT7BG-derived probe fragment protected by BBB + ARE mRNA; (A) pSP6 α 133-derived probe fragment protected by α -globin mRNA. Times correspond to minutes after serum stimulation. Beside the autoradiograms is a semilogarithmic plot of the decay of BBF Δ ARE mRNA (dashed/dotted line) and BBB + ARE mRNA (solid line).

the endogenous mouse *c-fos* message. These findings indicate that in serum-stimulated fibroblasts, actinomycin D inhibits decay mediated by the *c-fos* ARE but has little effect on degradation involving the instability determinant(s) present in the *c-fos* protein-coding region. This differential inhibition may be evidence that the ARE and the *c-fos* coding-region determinant are targets of two distinct cellular pathways for mRNA degradation. Alternatively, both determinants might be targeted by the same degradation pathway, and actinomycin D might inhibit ARE-mediated degradation selectively by site-specific intercalation into *c-fos* mRNA.

To distinguish between these possibilities, we examined the decay of BBF and BFB mRNA after treatment of serum-stimulated cells with 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB), a transcription inhibitor that functions by interacting specifically with the RNA polymerase II transcription apparatus rather than by intercalating into DNA [Zandomeni et al. 1982; Maderious and Chen-Kiang 1984]. Like actinomycin D, DRB gradually inhibited decay of the BBF transcript but had little effect on the degradation of BFB mRNA, which decayed with a uniform half-life of 25 ± 4 min in the

presence of DRB (Fig. 7, bottom). Because two transcription inhibitors with different modes of action both have the same differential effect on BBF and BFB mRNA decay, we conclude that the *c-fos* ARE and the *c-fos*-coding-region determinant direct rapid mRNA degradation by two distinct cellular pathways and that the degradation pathway that targets the ARE is transcription dependent in serum-stimulated cells.

Discussion

Our findings show for the first time that *c-fos* mRNA is targeted for rapid degradation by two distinct cellular pathways, each of which recognizes a different instability determinant within the *c-fos* transcript. One determinant of *c-fos* mRNA instability is the ARE within the *c-fos* 3'-UTR (Fig. 1). Insertion of this sequence into the 3'-UTR of β -globin mRNA reduces the half-life of that message from >24 hr down to only 37 min. Remarkably, this element is not necessary for *c-fos* mRNA instability, and its deletion has only a small effect on the half-life of the *c-fos* transcript.

We have identified a second, functionally independent

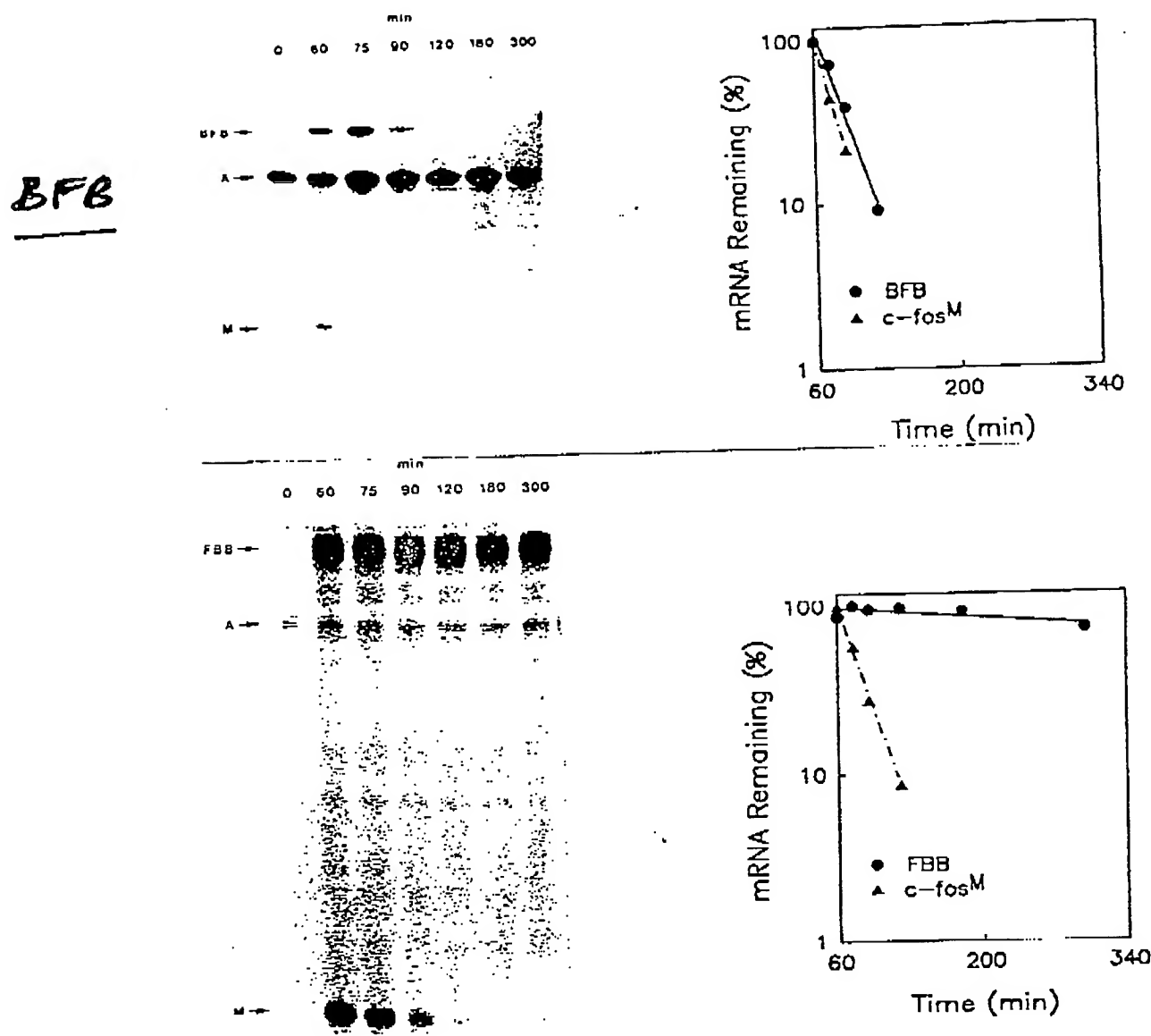


Figure 6. Decay of BFB and FBB mRNA. NIH-3T3 cells were transiently transfected with pSV α 1 and either pBFB (top) or pFBB (bottom). Total cytoplasmic RNA was isolated at time intervals after serum stimulation and analyzed by RNase protection and gel electrophoresis. (BFB) pSP6BFB-derived probe fragment protected by BFB mRNA; (FBB) pT7BG-derived probe fragment protected by FBB mRNA; (M) pSP6 α 133-derived probe fragment protected by mouse *c-fos* mRNA; (A) pSP6 α 133-derived probe fragment protected by α -globin mRNA. Times correspond to minutes after serum stimulation. Beside each autoradiogram is a semilogarithmic plot of the decay of BFB or FBB mRNA (solid line) and mouse *c-fos* mRNA (*c-fos*^M, dashed/dotted line).

determinant of *c-fos* mRNA instability within the protein-coding region of the message (Fig. 1). Replacement of the coding region of β -globin mRNA with the corresponding *c-fos* segment results in a dramatically reduced half-life of just 17 min. The presence of a destabilizing element in the *c-fos* protein-coding segment explains why deletion of the ARE or of the entire 3'-UTR has little effect on the decay rate of *c-fos* mRNA. Indeed, our data suggest that degradation mediated by this coding

region determinant is the primary pathway for *c-fos* mRNA decay. Recently, Kabaick and Housman (1988) have reported corroborating evidence that structural elements outside of the 3'-UTR contribute to *c-fos* mRNA instability.

Our data indicate that the ARE and the determinant(s) of instability in the *c-fos* coding region are each the target of a different cellular pathway for mRNA degradation. The *c-fos* coding region contains neither a long

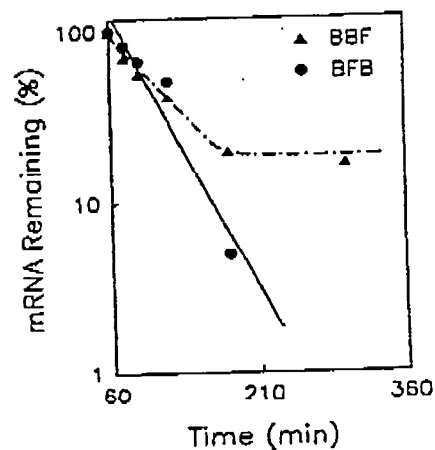
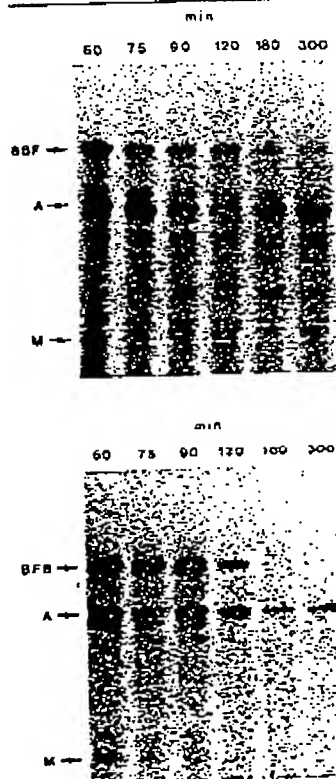
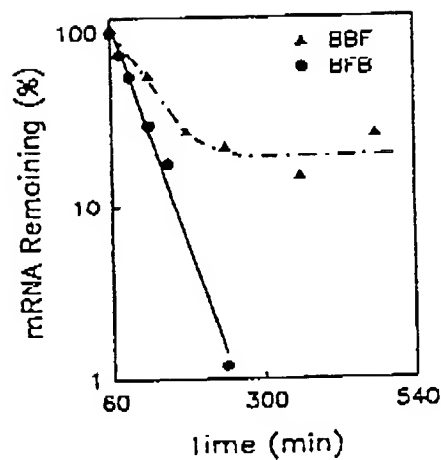
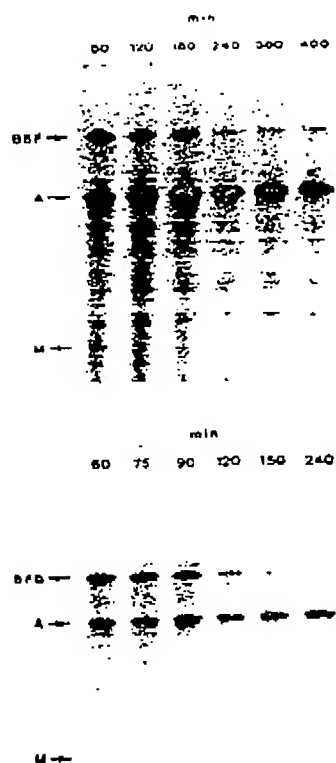


Figure 7. (See facing page for legend).

AU-rich segment nor the pentanucleotide AUUUA found in all known AREs. Moreover, treatment of serum-stimulated cells with either of two transcription inhibitors (DRB or actinomycin D) gradually blocks degradation mediated by the *c-fos* ARE but has little effect on the decay pathway for which the *c-fos* protein-coding region is a target.

Previous studies have shown that the *c-fos* transcript is stabilized by treatment of cells with translation inhibitors (Greenberg et al. 1986a), which could act by blocking synthesis of a labile protein necessary for *c-fos* mRNA degradation and/or by disrupting the normal polysomal structure of the *c-fos* message. Because DRB and actinomycin D inhibit transcription by entirely different mechanisms and because DRB is not thought to bind RNA, our findings indicate that the ARE-dependent mRNA degradation pathway requires synthesis of a labile gene product whose activity is lost within 3 hr after transcription is blocked. This unstable gene product apparently is not required for the degradation pathway that targets the *c-fos* coding-region determinant. If this gene product is a protein, it might help to explain the stabilizing effect of translation inhibitors on *c-fos* mRNA; it would also imply that the message encoding this protein is itself highly labile in serum-stimulated cells, as the effect on protein synthesis of inhibiting synthesis of a stable transcript would not be apparent for many hours.

Our finding that deletion of the *c-fos* ARE hardly affects the half-life of the human *c-fos* message differs from previous studies of mouse *c-fos* mRNA (Fort et al. 1987; Kahmsdorf et al. 1987). In those experiments, the decay rate of the mouse *c-fos* transcript slowed significantly upon removal of large 3'-UTR segments containing the ARE. The difference between our results and those of others might be explained by the way the experiments were carried out. Previous studies of mouse *c-fos* mRNA decay used actinomycin D in actively growing cells to block constitutive transcription of *c-fos* from a heterologous promoter. In contrast, to ensure physiological conditions relevant to normal *c-fos* gene expression, our studies have been performed in growth-factor-stimulated cells. Moreover, we have monitored mRNA decay in the absence of transcription inhibitors to avoid the perturbation of mRNA half-life that can occur in cells treated with such inhibitors (see above; Mullner and Kuhn 1988). Finally, in creating hybrid *c-fos*/ β -globin transcripts, we have been careful to minimize translational perturbations that could affect mRNA decay. For example, to avoid disrupting translation initiation signals (Kozak 1987), we have fused the *c-fos* and β -globin transcripts at sites downstream of the second codon. Furthermore, the chimeric transcripts examined

in the present study have been designed to provide ribosomal coverage for all mRNA segments that normally are translated and to prevent translation of normally untranslated RNA segments.

The method described here for studying *c-fos* mRNA decay has additional features that deserve emphasis. The observation that the lifetime of *c-fos* mRNA is unnaturally prolonged in cells transfected with an excessive amount of *c-fos* DNA is crucial. It has led us to identify transient-transfection conditions under which wild-type *c-fos* mRNA produced from the transfected gene decays at the same rapid rate as the endogenous *c-fos* message. Furthermore, because fusion of the *c-fos* promoter to other transcription units allows transient synthesis of heterologous mRNA in response to serum addition (see below), it has been feasible to examine the decay of hybrid β -globin/*c-fos* messages under conditions identical to those employed for monitoring decay of the *c-fos* transcript itself. The strategy of swapping segments between the labile *c-fos* transcript and the stable β -globin message has permitted the identification of destabilizing determinants within *c-fos* mRNA that function in growth-factor-stimulated cells. This same approach should facilitate precise mapping of the sequence elements within the ARE and coding region that are essential for rapid degradation of the *c-fos* message. It should also allow identification of the determinants that render other growth-factor-inducible mRNAs unstable.

Studying *c-fos* mRNA decay in this manner requires that transcription end within minutes after growth-factor induction. Degradation of the *c-fos* message can then be monitored in the absence of further mRNA synthesis. Our experiments indicate that, as for the endogenous *c-fos* gene, heterologous gene transcription driven by the *c-fos* promoter is turned off shortly after growth-factor stimulation. This is indicated by nuclear run-on analysis of transcription of the BBB and BBF genes (V. Rivera and M.E. Greenberg, unpubl.) and by the finding that cytoplasmic accumulation of the BBB, BFB, BBF, and BBB+ARE transcripts ceases within 60 min after serum induction. Because the only *c-fos* element shared by all of these chimeras is the *c-fos* promoter, the *c-fos* gene segment located between 14 and 711 bp upstream of the transcription initiation site must contain all of the cis-acting DNA sequence information necessary for stimulation and repression of transcription in response to growth factors. We conclude that in cells transfected with the BBB gene, the high level of β -globin mRNA present as late as 24 hr after growth-factor stimulation reflects the very long lifetime of β -globin mRNA and not a loss of transcriptional repression.

The existence of two pathways for rapid degradation

Figure 7. Decay of BBF and BFB mRNA in cells treated with transcription inhibitors. NIH-3T3 cells were transiently transfected with pSVol and either pBBF or pBFB. After serum stimulation for 30 min, actinomycin D (top; 10 μ g/ml) or DRB (bottom; 30 μ g/ml) was added, and total cytoplasmic RNA was isolated at time intervals and analyzed by RNase protection and gel electrophoresis. (BBF) pT7BG-derived probe fragment protected by BBF mRNA; (BFB) pSP6BFB-derived probe fragment protected by BFB mRNA; (M) pSP6fos-derived probe fragment protected by mouse *c-fos* mRNA. (A) pSP6 α 133-derived probe fragment protected by α -globin mRNA. Times correspond to minutes after serum stimulation. Beside each pair of autoradiograms is a semilogarithmic plot of the decay of BBF mRNA (dashed/dotted line) and BFB mRNA (solid line) in the presence of actinomycin D (top) or DRB (bottom).

of *c-fos* mRNA could serve important biological functions. For example, certain extracellular signals might activate or inhibit one decay pathway but not the other. If so, these signals could cause dramatic half-life changes for transcripts bearing a single instability determinant, without significantly affecting the half-lives of messages able to decay by more than one pathway. This would allow a greater degree of flexibility in a cell's response to its environment or state of growth. Interestingly, precise deletion of either the ARE or a sequence near the 3' end of the coding region appears to contribute to the oncogenic potential of the *c-fos* gene when it is expressed from a strong constitutive promoter [Meijlink et al. 1985]. Because overexpression of the *c-fos* proto-oncogene can lead to oncogenesis, multiple degradation pathways may have evolved to ensure rapid turnover of *c-fos* mRNA in a variety of cell states.

Materials and methods

Plasmid constructions

DNA linkers were purchased from New England Biolabs. Oligonucleotides used for site-directed mutagenesis were synthesized using an Applied Biosystems 381A instrument and purified by gel electrophoresis.

The α -globin reference plasmid pSV α 1 was constructed by subcloning a 1.80-kb *Bgl*II (filled in)-*Xba*I fragment from plasmid pSVHP α 2 [Chamay et al. 1984], comprising the human α 1-globin gene driven by the SV40 early promoter and enhancer, between the *Pst*I (filled in) and *Xba*I sites of plasmid pT7/T3 α -18 [BRL].

Plasmid pF711 [Treisman 1985] contains a complete genomic copy of the human *c-fos* gene, beginning 0.71 kb upstream of the transcription initiation site and ending 1.60 kb beyond the polyadenylation site.

Plasmid pF Δ 1, a pF711 derivative, was constructed in several steps as follows. A 27-bp *Bam*HI-*Hind*III fragment was deleted from the polylinker region of pF711 to generate plasmid pF99. An *Nco*I site and a *Bam*HI site were then introduced into pF99 at the promoter-proximal end of the *c-fos* ARE by oligonucleotide-directed mutagenesis [Nakamaye and Eckstein 1986] of a 4-bp segment located 623 bp downstream of the *c-fos* stop codon (CCATGAAAAC \rightarrow CCATGGATCC). Simultaneous introduction of a point mutation 704 bp downstream of the *c-fos* stop codon (CTTGAG \rightarrow CTCGAG) created an *Xho*I site at the promoter-distal end of the *c-fos* ARE and generated pF100. The 76-bp *Bam*HI-*Xho*I fragment of pF100 was then excised, and the ends were filled in and religated to generate plasmid pF Δ 1.

Plasmid pF101 was created by linearizing pF100 with *Xho*I, removing the protruding 5' ends with mung bean nuclease, and inserting a *Bgl*II linker (CGAGATCTCC) by the linker-tailing method [Lathe et al. 1984].

A nested set of deletions in the 3'-UTR of the human *c-fos* gene was constructed as follows. Bidirectional deletions were created from the *Bgl*II site of pF101 by exonuclease III/exonuclease VII (BRL) digestion followed by insertion of a *Bgl*II linker (CGAGATCTCC). The complete *c-fos* gene segment downstream of the ARE was then reintroduced into the resulting plasmids by replacing the *Bgl*II-*Eco*RI fragment containing the plasmid replication origin with the intact 3.34-kb *Bgl*II-*Eco*RI fragment of pF101, to generate plasmids pF Δ 2-pF Δ 6. End points were determined by analytical restriction enzyme digestions and agarose gel electrophoresis.

To generate plasmid pF Δ 7, a 0.46-kb *Hind*III fragment

flanking the *c-fos* gene was first excised from plasmid pF4, and the ends were filled in and religated. A *Hind*III linker (CCCAAGCTTGGG) was then inserted between the *Bcl*I site and proximal *Tth*1111 site of the resulting plasmid by the linker-tailing methods.

Plasmids carrying hybrid genes with segments of the rabbit β -globin gene and the human *c-fos* gene were constructed in several steps as follows.

A 2.05-kb *Bgl*II fragment containing a complete copy of the genomic rabbit β -globin transcription unit was excised from plasmid pUK4.7 by partial *Bgl*II digestion and substituted for the 1.32-kb *Bam*HI fragment of pSV α 1 to generate plasmid pSVB10. In pSVB10, the orientation of the β -globin gene was the same as that of the α -globin gene that it replaced.

Plasmid pFFB was created by replacing the 1.28-kb *Bgl*II-*Hind*III (filled in) fragment of pF Δ 6 with a 0.44-kb *Bgl*II-*Eco*RI (filled in) fragment of pSVB10 that contained the 3'-UTR of the rabbit α -globin gene.

To generate plasmid pBBB, a 2.66-kb *Nar*I (filled in)-*Bgl*II fragment of pFFB, extending from 13 bp upstream of the *c-fos* transcription initiation site to the β -globin/*c-fos* junction, was replaced with a 1.21-kb *Pvu*II-*Bgl*II fragment of pSVB10, extending from 9 bp upstream of the rabbit β -globin transcription initiation site to the end of the β -globin coding region.

Plasmid pBBB3 was generated by substituting a portion of the pUC18 polylinker region (*Kpn*I-*Hind*III) for the 0.46-kb *Kpn*I-*Hind*III fragment downstream of the polyadenylation site of pBBB.

Plasmid pBBB4 was generated by oligonucleotide-directed mutagenesis of pBBB3, into which an *Mlu*I site was introduced by changing the sequence of the third and fourth β -globin codons from CATCTG to CACGGC.

Plasmid pF106 was constructed in several steps as follows. A 0.46-kb *Hind*III fragment was deleted from pF711, and a *Bgl*II linker (GGAGATCTCC) was inserted into the *Nae*I site near the beginning of the *c-fos* 3'-UTR to generate plasmid pF104. A *Hinc*II site downstream of the *c-fos* polyadenylation site in pF104 was destroyed by insertion of a *Hind*III linker (CCCAAGCTTGGG), and an *Mlu*I site was created by oligonucleotide-directed mutagenesis of the third, fourth, and fifth *c-fos* codons (TTCTCGGGC \rightarrow TACGCGTGC), to generate plasmid pF106.

To construct plasmid pBBF, a 1.91-kb *Bgl*II-*Hind*III (filled in) fragment of pF104 was substituted for the 0.44-kb *Bgl*II-*Kpn*I (filled in) fragment of pBBB. To construct plasmid pBBF, a 2.46-kb *Mlu*I-*Bgl*II fragment of pF106 was substituted for the 1.14-kb *Mlu*I-*Bgl*II fragment of pBBB4. To construct plasmid pFBB, a 263-bp *Bss*HII-*Mlu*I fragment of pF106 was substituted for the 161-bp *Bss*HII-*Mlu*I fragment of pBBB4.

Plasmid pBBF Δ ARE is a deletion derivative of pBBF and was constructed from pBBB3 by replacing the 0.45-kb *Bgl*II (filled in)-*Hind*III fragment with the 2.34-kb *Nae*I-*Hind*III fragment of pF Δ 1.

The 83-bp *Bam*HI-*Bgl*II fragment of pF101 encoding the *c-fos* ARE was subcloned into *Bgl*II-linearized pBBB to generate plasmid pBBB-ARE. The transcription orientation of the insert was the same as that in plasmid pF101.

Plasmids for generation of complementary-strand RNA probes were prepared as follows. Plasmid pT7BG was constructed by deleting a 1.17-kb *Eco*RI-*Bam*HI fragment from pSVB10. Plasmid pSP6BFB was constructed by inserting a 266-bp *Nar*I-*Hinc*II fragment of pBBB between the *Acc*I and *Hind*III (filled in) sites of pGEM3 [Promega].

Cell culture and transfection

Mouse NIH-3T3 cells were passaged in Dulbecco's modified

Eagle's medium (DMEM) with 10% calf serum. Cells were split to a density of 2×10^6 /100-mm dish 18 hr before transfection by the calcium phosphate technique (Treisman 1985). Transfection mixtures contained 2–3 μ g of the test plasmid, 1 μ g of the internal control α -globin plasmid (pSV α 1), and 17 μ g of carrier plasmid (pT7/T3 α 19, BRL). After exposure to the plasmid precipitate for 12–16 hr, cells were serum-starved in DMEM/0.5% calf serum for 26–30 hr and then stimulated with DMEM/15% fetal calf serum as described previously (Greenberg et al. 1987).

Analysis of mRNA decay

Total cytoplasmic RNA was isolated at various times after serum stimulation, as described previously (Greenberg and Ziff 1984; Greenberg et al. 1986b). Transcripts derived from transfected DNA were detected by RNase protection analysis (Treisman 1985) of equal amounts of total cytoplasmic RNA, using complementary RNA probes. Human and mouse *c-fos* mRNA and certain derivatives thereof were detected with a 588-nucleotide RNA probe derived from *Eco*RI-linearized template plasmid pSP6fos (Treisman 1985). This probe is complementary to the 5'-terminal segment of the human transcript and yields a protected fragment of 296 nucleotides or 65 nucleotides for human or mouse *c-fos* mRNA, respectively. Human α -globin mRNA was detected with a 270-nucleotide RNA probe that was derived from *Bam*HI-linearized plasmid pSP6 α 133 (Chamay et al. 1984) and that hybridized to a 133-nucleotide α -globin mRNA segment within the first exon. Rabbit α -globin mRNA and certain derivatives thereof were detected with a 200-nucleotide RNA probe that was derived from *Nco*I-linearized pT7BG and that hybridized to a 188-nucleotide rabbit α -globin mRNA segment within the second exon. BFB mRNA was detected with a 280-nucleotide RNA probe that was derived from *Bam*HI-linearized pSP6BFB and that hybridized to a 180-nucleotide BFB mRNA segment within the first exon.

To measure mRNA decay rates, data from autoradiograms were quantitated by densitometry on an LKB Ultrascan XL instrument. For each sample, the concentration of mRNA derived from the transfected *c-fos* gene or derivative thereof was normalized to the α -globin mRNA internal standard. The concentration of endogenous mouse *c-fos* mRNA was normalized to the concentration of total cytoplasmic RNA. mRNA half-lives were then determined by least-squares analysis of semilogarithmic plots of normalized mRNA concentration as a function of time. Half-life errors were estimated from the standard deviation of the slope of each plot. Only data from samples isolated ≥ 60 min after serum stimulation were used to ensure that mRNA synthesis directed by the *c-fos* promoter had ceased.

Acknowledgments

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MESSENGER RNA DEGRADATION in eukaryotic cells is a regulated process that provides a powerful means for controlling gene expression (for recent reviews, see Refs 1 and 2). This review will examine how a group of AREs found in the 3' UTR of mRNAs target those molecules for rapid and selective degradation. ARE-directed mRNA degradation is linked to cell transformation and oncogenesis, and so elucidation of its mechanism of action is important. For example, deletion of the ARE from the 3' UTR of *c-fos* mRNA results in the conversion of the *c-fos* proto-oncogene to an oncogene (reviewed in Ref. 3). Moreover, the ARE within the 3' UTR of interleukin 3 (IL-3) mRNA is required for inhibition of the growth of autocrine tumor mast cells by cyclosporin A, an immunosuppressant⁴.

Key sequence features

The conservation of an adenylate/uridylylate (AU)-rich region was first noted for genes coding for cytokines⁵, which are inflammatory mediators. Subsequently, using *c-fos*/β-globin gene fusions, the *c-fos* 3' UTR, which has an ARE, was implicated in mRNA instability⁶. The first direct evidence that the ARE can function as a potent mRNA destabilizing element came from a study in which a conserved region of 51 nucleotides containing AUUUA motifs from the 3' UTR of human granulocyte-macrophage-colony-stimulating factor (GM-CSF) mRNA was inserted into the 3' UTR of β-globin mRNA; the otherwise stable β-globin mRNA was destabilized⁷. More recent experiments have shown that a number of AU-rich sequences bearing AUUUA motifs from the *c-fos*, *c-myc*, *nur77*, *junB*, β-interferon (β-IFN) and IL-3 mRNAs also function as RNA destabilizing elements (Table I).

AREs range in size from 50 to 150 nucleotides. Generally, they contain multiple copies of the pentanucleotide, AUUUA, and have a high content of U and sometimes also A residues (Table I). As different AREs vary considerably in their size and sequence, it has never been clear which parts of the sequence are functionally important. Can we assume that the presence of AUUUA motifs in an AU-rich region designates a functional ARE? Is there a minimal core

AU-rich elements: characterization and importance in mRNA degradation

Chyi-Ying A. Chen and Ann-Bin Shyu

Adenylate/uridylylate-rich elements (AREs) are found in the 3' untranslated region (UTR) of many messenger RNAs (mRNAs) that code for proto-oncogenes, nuclear transcription factors and cytokines. They represent the most common determinant of RNA stability in mammalian cells. Moreover, ARE-directed mRNA degradation is influenced by many exogenous factors, including phorbol esters, calcium ionophores, cytokines and transcription inhibitors. These observations suggest that AREs play a critical role in the regulation of gene expression during cell growth and differentiation, and in the immune response.

consensus sequence that is necessary for the destabilizing function of AREs? Do all AREs belong to one class, or can they be subdivided into distinct classes?

Several approaches have recently been taken to address these critical questions. First, extensive mutagenesis has been used to determine the key sequence features of the *c-fos*⁸ and IL-3⁹ AREs. Second, the destabilizing abilities of at least eight other AU-rich sequences from the 3' UTRs of several labile early response gene (ERG) mRNAs¹⁰ and GM-CSF mRNA⁷ have been determined. Third, a series of synthetic AU-rich sequences have been created and tested for their ability to destabilize stable reporter mRNAs^{11,12}. Several important observations have been made. (1) The presence of AUUUA motif(s), even in an AU-rich region, does not guarantee a destabilizing function of an ARE. Several AU-rich sequences from the labile ERG mRNAs, such as *c-jun* #1, *krox20* #1 and #2, and *zif268* #1 (Table I), possess the two general features of the ARE, yet do not confer instability on the stable β-globin mRNA¹⁰. (2) AUUUA motifs play a critical role in determining the destabilizing ability of the *c-fos*, GM-CSF and IL-3 AREs. For example, the three AUUUA motifs in the *c-fos* ARE appear to function independently. Together, they have an additive effect on RNA destabilization, as successive mutagenesis of each AUUUA motif leads to a gradual loss of destabilizing function⁸.

(3) By use of synthetic AU-rich sequences, a nonamer with an AUUUA pentanucleotide core, UUAUUUA(U/A)-(U/A), gave a modest destabilizing effect^{11,12}. Interestingly, it appears that cytokine AREs such as the GM-CSF, IL-3, and β-IFN AREs have at least two overlapping copies of this nonamer within a highly U-rich region (Table I), whereas all known functional AREs from ERG mRNAs, such as *c-myc*, *junB*, *c-jun* #2, and *nur77*, do not contain the nonamer. The exception to this is *c-fos* ARE, which has one copy. However, several single point mutations that alter the nonamer present in the *c-fos* ARE have little effect on its RNA destabilizing function⁸. (4) Analysis of the *c-fos* ARE has demonstrated the importance of a high percentage of U residues. The 69 nucleotide *c-fos* ARE contains two structurally distinct and functionally interdependent domains. Domain I has three copies of the AUUUA motif and alone it has a potent destabilizing ability which can be enhanced by the U-rich domain II⁸. Reduction of the U content of domain I by changes of U to G or U to C dramatically impairs its RNA destabilizing function. This effect can be rescued by fusing back the U-rich domain II immediately downstream of the mutated domain I (Ref. 8). Moreover, several non-functional AU-rich sequences can be converted to potent destabilizing elements by placing the *c-fos* U-rich domain II immediately downstream of them¹⁰.

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Table I. Features of the AU-rich sequences from the 3' UTRs and mRNAs^a

AU-rich sequence	Length of AU-rich sequence (nucleotides)	Adenine (%)	Uridine (%)	No. of AUUUA motifs ^b	No. of UUAUUUA(U/A)(U/A) nonamers ^c	Destabilizing effect ^d	...s)
<i>c-fos</i>	69	23	65	3	1	+	6, 10
<i>c-jun</i>							
#1	158	33	37	2	0	-	10
#2	146	25	47	0	0	+	10
<i>junB</i>							
#1	70	30	51	1	0	+	10
#2	104	24	41	1	0	+	10
<i>c-myc</i>	138	35	47	2	0	+	10, 40,
41							
<i>krox20</i>							
#1	70	23	54	1	0	-	10
#2	63	44	63	2	0	-	10
<i>nu77</i>	84	32	49	3	0	+	10
<i>zif268</i>							
#1	86	47	34	2	0	-	10
#2	96	20	48	0	0	+	10
#3	107	26	48	0	0	-	10
<i>GM-CSF</i>	51	37	63	7	3	+	7, 10
<i>IL-3</i>	55	25	62	6	2	+	9
<i>β-IFN</i>	60	33	58	2	2	+	42

^aAbbreviations used are as described in the text.^bIncludes overlapping AUUUA motifs.^cIncludes overlapping UUAUUUA(U/A)(U/A) motifs.^d+, at least threefold destabilizing effect on the reporter mRNA is detected; -, less than twofold effect.

Is AUUUA an absolute sequence requirement for the destabilizing function of an ARE? The study of two functional AREs, one from the *c-jun* 3' UTR and the other from the *zif268* 3' UTR (*c-jun* #2 and *zif268* #2 in Table I), that contain no AUUUA motifs, has provided a clue¹⁰. The non-AUUUA ARE from *c-jun* has a potent destabilizing function that directs rapid mRNA decay with a biphasic pattern that is characteristic of the *c-fos* ARE (see below). It is now clear that the AUUUA pentanucleotide need not be an integral part of a functional ARE.

Taken together, these studies suggest that each ARE represents a combination of functionally and structurally distinct sequence motifs or domains, such as AUUUA motifs, UUAUUUA(U/A)(U/A) nonamers, U stretches and/or a U-rich domain. It is the combination of these sequence features within an ARE that determines the ultimate destabilizing function of that particular ARE. Table II shows a rough classification of AREs¹³ and a summary of some of the critical sequence features and functional properties of representative AREs.

Deadenylation: the first step in ARE-directed mRNA degradation

The use of the serum-inducible promoter from *c-fos* to drive transient mRNA synthesis, following stimulation of quiescent fibroblasts by serum, provided the first evidence that AREs facilitate rapid deadenylation as the first step in mRNA degradation^{14,15}. These

transcriptional pulse-chase experiments showed that the *c-fos* ARE mediates rapid mRNA decay with a biphasic pattern^{8,15}. During the first phase, poly(A) tails are synchronously and rapidly shortened to a size of 30–60 nucleotides, while the mRNA concentration remains fairly constant. In the second phase, the transcribed portion of the mRNA is degraded with apparent first-order kinetics (Fig. 1).

Mutations within the *c-fos* ARE alter the kinetics of degradation and corroborate that deadenylation precedes decay of the transcribed mRNA⁸. For instance, the AUUUA motifs facilitate degradation of the RNA body, while the U-rich domain promotes deadenylation and enhances the destabilizing function of the AUUUA motifs⁸. These results also highlight the point that the rates of ARE-directed decay are controlled by sequence features of the ARE.

The generality of this apparent 'two-step' decay mechanism among various AREs has also been tested^{16,17}. It appears that all functional AREs mediate deadenylation as the first step in mRNA decay, although different classes of ARE exhibit different reaction kinetics (Table II). For example, the GM-CSF ARE, which is classified as a class II AUUUA-containing ARE, mediates asynchronous deadenylation with the formation of fully deadenylated intermediates, consistent with the possibility that ribonucleolytic digestion of poly(A) tails is processive¹³ (Fig. 1). By contrast, class I AUUUA-containing

(e.g. *c-fos* ARE) and non-AUUUA (e.g. *c-jun* #2 ARE) AREs mediate a synchronous poly(A) shortening which proceeds at the same rate, indicating that ribonucleolytic digestion of poly(A) tails is distributive or non-processive^{8,10}. Since in yeast, deadenylation also serves as a critical first step in the decay of both stable and labile mRNAs¹⁸, it is possible that different RNA destabilizing elements in yeast mRNAs direct deadenylation by different kinetics. This could be achieved, for example, by modulating the processivity at which a single ribonuclease functions.

Translational coupling

Many experiments have shown that ongoing translation of an mRNA, either directly or indirectly, influences its half-life². Is ARE-directed mRNA decay coupled to protein translation and if so, by what mechanism? One study, using an iron response element (IRE) to regulate translation, suggests that the destabilization of a heterologous mRNA by the *c-fos* ARE is independent of on-going translation of the mRNA¹⁶. By contrast, two other studies suggest that mRNA decay directed by the GM-CSF ARE is coupled to translation^{17,18}. These studies are provocative and suggest a different requirement for coupling the RNA destabilizing function to translation by the two AREs. However, different cell types, reporter mRNAs, and experimental conditions were used in each case, and so it is not known whether the two AREs

would exhibit the observed patterns of behavior under the same genetic background and physiological conditions.

In an attempt to clarify the issue, two direct comparisons have been made. In one study¹⁹, an IRE was used to regulate translation in mouse NIH3T3 cells. The IRE was placed within the 5' UTR of an α -globin mRNA that contained either the *c-fos* or the GM-CSF ARE in its 3' UTR. When translation initiation was blocked by iron chelator-induced binding of iron regulatory protein to the IRE, there was no apparent decay of the mRNA bearing either ARE following transcription inhibition by actinomycin D in NIH3T3 growing cells. This study showed that the destabilization of a heterologous mRNA by the *c-fos* or GM-CSF ARE is dependent upon ongoing translation of the mRNA by ribosomes. By contrast, the other comparison¹⁵ showed that mRNA decay directed by the *c-fos* or GM-CSF ARE can be uncoupled from translation. The effect of a block in translation initiation on the half-life of β -globin mRNAs that contain in their 3' UTRs either the *c-fos* or GM-CSF ARE, was determined in NIH3T3 cells by driving β -globin gene expression with the transiently inducible *c-fos* promoter. The block was brought about by inserting a stable stem-loop structure close to the 5' cap of the mRNA. In spite of the efficient translation block as demonstrated by polysome profile studies, the two AREs retained their full destabilizing ability.

One possibility that may reconcile the apparent discrepancy between these two studies is that the growth status of the cell (i.e. growing cells versus cells in G0 to G1 transition) may alter the requirement for translation in order for AREs to act as a destabilizing element. For example, in NIH3T3 cells, serum induction may lead to activation of an alternative pathway that is independent of the translational status of the mRNA carrying the ARE. While further experiments are required to resolve this discrepancy, it is clear that the destabilizing function of AREs is not obligatorily coupled to translation.

ARE-binding proteins

A detailed understanding of how various AREs function will require a rigorous investigation of the *trans*-acting factors (binding proteins) that interact with the AREs. Following the first

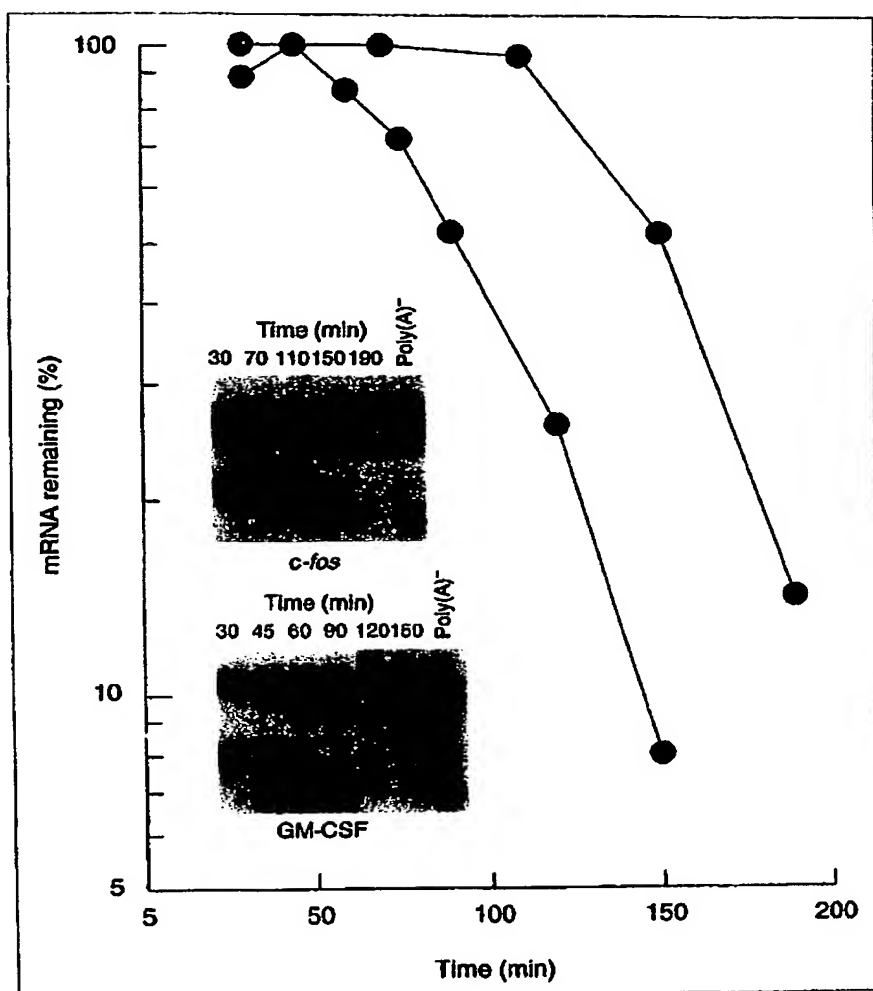


Table II. Sequence features and functional properties of the three classes of ARE*

ARE	Example	Sequence features	Decay kinetics	Deadenylation	Sensitivity to actinomycin D	Coupling to translation
AUUUA-containing class I	<i>c-fos</i>	Has 1-3 copies of scattered AUUUA motifs coupled with a nearby U-rich region or U stretch	Biphasic, deadenylation precedes decay of the RNA body	Synchronous, results in decay intermediates with poly(A) tails of 30-60 nucleotides	Sensitive	Not necessary
class II	GM-CSF	Has at least two overlapping copies of the nonamer UUAUUUA(U/A)(U/A) in a U-rich region	Biphasic, deadenylation precedes decay of the RNA body	Asynchronous, results in poly(A)-decay intermediates	Sensitive	Not necessary
Non-AUUUA	<i>c-jun</i>	Has a U-rich region and other features (?)	Biphasic, deadenylation precedes decay of the RNA body	Synchronous, results in decay intermediates with poly(A) tails of 30-60 nucleotides	Not sensitive	Not necessary

*Abbreviations used are as described in the text.

between both compartments (see below). Second, the binding activity of ARE-binding proteins can correlate inversely or proportionally with the stability of mRNAs that have an ARE. Third, like other RNA processing events, the ARE may trigger mRNA decay through formation of a complex of proteins on the ARE.

Recently, the genes of several mammalian proteins having ARE- or U-rich-sequence binding activity have been cloned. These include the genes for heterogeneous nuclear ribonucleoprotein (hnRNP) A1 (Refs 23, 24), hnRNP C (Ref. 23), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Ref. 25), HeI-N1 (a human neuron-specific RNA-binding protein; Ref. 26) and, more recently, a 38 kDa protein termed Aul (Ref. 27), a 32 kDa protein with enoyl-CoA hydratase activity (Ref. 28) and hnRNP A0 (Ref. 29).

A few recent studies may shed some light on the involvement of certain ARE-binding proteins in ARE-mediated mRNA decay. Two commonly used transcription inhibitors, actinomycin D and 5,6-dichloro-1- β -D-ribofuranosyl-benzimidazole (DRB), can specifically lead to complete stabilization of β -globin mRNA bearing the *c-fos* or GM-CSF ARE in NIH3T3 cells¹⁵. Interestingly, in cells treated with either one of these two drugs for 3h, there is a several-fold increase in the cytoplasmic concentration of at least two proteins (AU-A and hnRNP A1) that have high affinity for U-rich sequences^{24,30}. More importantly, these two proteins also bind to the *c-fos* and GM-CSF AREs *in vitro*²⁴. It is tempting to speculate that, following arrest of nuclear transcription, certain hnRNP proteins (and probably other ARE-binding proteins) that can shuttle between the nucleus and cytoplasm are retained

or transported to the cytoplasm. This might lead to a temporary increase in the cytoplasmic concentration of these proteins, which could, in turn, result in competitive displacement of the other cognate ARE-binding proteins, or, alternatively, could change the existing cytoplasmic equilibrium of the binding between these proteins and their AREs. As a result, the ARE-protein complexes that are necessary for ARE-directed mRNA decay might be disrupted.

Regulation and signaling pathways

Several lines of evidence suggest that the two classes of AUUUA-containing AREs may be differentially regulated. In a monocytic tumor cell line, *c-fos* and *c-myc* 3' UTRs (which contain class I AREs) destabilized a *neo* reporter mRNA whereas the GM-CSF 3' UTR (containing a class II ARE) did not³¹. A similar discrimination between the two types of AREs has also been suggested in two other studies. Stimulation of quiescent primary T cells with antibodies to CD3/CD28 receptors specifically stabilizes four lymphokine mRNAs including GM-CSF mRNA, while *c-fos* and *c-myc* mRNA remain labile³². The IL-3 ARE, which has class II characteristics, is required in a mast tumor cell line for the destabilization of IL-3 mRNA by cyclosporin A (Ref. 4). In contrast, the stability of IL-4 and IL-6 mRNAs, which contain the class I-like AREs, remain unchanged in the same cells. Moreover, further experiments showed that the role of IL-3 AREs cannot be substituted for by the class I AREs from either *c-fos* or *c-myc*³³. Although a complete understanding of the underlying mechanisms awaits further experiments, it is possible that sequence differences between the two classes of AUUUA-containing AREs

may confer differential regulation of their destabilizing function in a cell-type specific manner.

The role of signal transduction in the control of ARE-directed mRNA decay has not been widely explored. Several previous studies have shown that treatment of lymphoid cells with phorbol esters, calcium ionophores or interleukins increases the steady-state level of certain cytokine mRNAs that contain AREs^{7,32,34,35}. Ca²⁺ ionophores like A23187 specifically stabilize IL-3 mRNA in cultured mast cells³⁶. A23187 also stabilizes GM-CSF mRNA at least tenfold in a thymoma cell line³⁷. In phorbol-ester-stimulated T cells, there is a correlation between stabilization of lymphokine mRNAs and loss of the ARE-binding activity of the AU-B ARE-binding protein²². Interestingly, AU-B interacts with the AU-rich sequence found in the 3' UTR of GM-CSF mRNA but not with *c-myc* mRNA. Taken together, these observations point to the possible differential regulation of ARE-specific mRNA degradation by Ca²⁺-dependent protein kinase C pathways. However, in view of the complex responses of cells to phorbol esters and to protein kinase C activation, it is unlikely that a single pathway or mechanism exists for regulating all instances of mRNA stabilization. The change of ARE destabilizing ability in response to calcium ionophores and phorbol esters is probably cell-specific, ARE-specific or both.

ARE-directed mRNA degradation pathway(s)

On the basis of the studies discussed above, we propose a general model for ARE-mediated mRNA degradation (Fig. 2), in which the common initial step of deadenylation can proceed

in two different ways. These two kinetically distinct deadenylation events produce two different mRNA intermediates. Whether the subsequent decay of these two types of intermediates carried out by the same exo- or endonucleases remains to be addressed. The difference in organization of some sequence features, such as the spacing among AUUUA pentanucleotides, the nonamer, and U-rich region, could result in the formation of different RNA-protein complexes or different ways of organizing the same *trans*-acting factors. These distinct ribonucleoprotein complexes could directly influence and modulate the processivity of a ribonuclease or recruit kinetically distinct ribonucleases. Alternatively, they could influence the stability of the poly(A)-poly(A)-binding-protein complex which, in turn, could make poly(A) tails more susceptible to ribonuclease attack³⁸.

Perspectives

Although our knowledge of ARE-directed mRNA decay is far from complete, progress has been made towards identifying the key sequence features of AREs and the cellular factors that participate in ARE-directed mRNA degradation. Clearly, many critical questions remain to be answered. In particular, identifying and characterizing the cognate ARE-mRNP complexes and the cytoplasmic ribonuclease(s) responsible for degrading both poly(A) tails and the mRNA body will be necessary. The existence of multiple ARE-binding proteins, often coexisting within a single cell, provides an additional level of complexity. In light of recent new information on mRNA degradation in yeast, in which deadenylation leads to decapping of the mRNA followed by 5' to 3' exonuclease digestion (reviewed in Ref. 2), one priority is to identify the decay intermediates that are formed following deadenylation in the ARE-mediated mRNA degradation. Additionally, the identification of a nuclease dependent on poly(A)-binding protein that appears to catalyse the removal of mRNA poly(A) tails in yeast³⁹ suggests that an analogous nuclease(s) might catalyse the two distinct deadenylation reactions directed by AREs.

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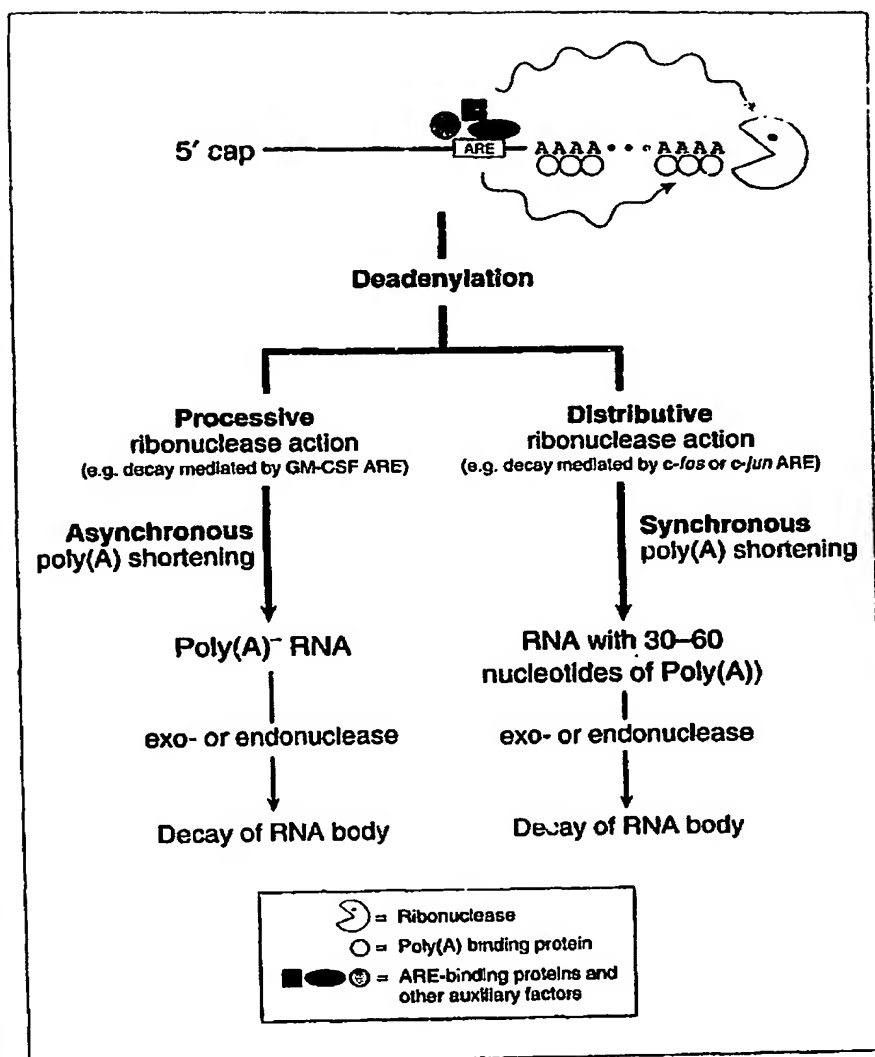


Figure 2

A general model for adenylate/uridylate-rich element (ARE)-mediated mRNA degradation showing two deadenylation pathways with distinct kinetics. For decay mediated by the class I AUUUA-containing ARE and the non-AUUUA ARE, the mRNA population undergoes synchronous poly(A) shortening and is deadenylated at the same rate, implying the action of a distributive or non-processive ribonucleolytic digestion of poly(A) tails. In contrast, the population of class II AUUUA-containing ARE mRNAs is deadenylated asynchronously, with the formation of fully deadenylated intermediates, consistent with the action of a processive ribonucleolytic digestion of poly(A) tails. Messenger ribonucleoprotein (mRNP) complexes involving different ARE-binding proteins that are formed on distinct AREs might influence deadenylation, for example, by modulating the rate at which a single ribonuclease functions or by recruiting kinetically distinct ribonucleases. Alternatively, they might exert their effect through changing the stability of poly(A)-poly(A) binding protein complexes.

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PROTEIN PHOSPHORYLATION IS a rapidly reversible reaction that has become a general mechanism for the regulation of intracellular events in response to environmental changes^{1,2}. The genes encoding a large number of serine/threonine-specific, tyrosine-specific, or dual-specificity protein kinases have been cloned and compared³, and it is estimated that hundreds of novel protein kinases are yet to be identified⁴. On the basis of sequence conservation at the catalytic domain, it is clear that both protein Ser/Thr kinases and Tyr kinases evolved from a common ancestor, then diverged to recognize distinct sets of substrates in the cell, thereby regulating distinct cellular responses. How does each of the several hundred protein kinases in a single cell find a unique set of protein substrates and thus maintain specificity in the many diverse signalling cascades? Here, we discuss the role of protein-protein interaction domains and the catalytic domains of protein tyrosine kinases (PTKs) in determining substrate specificity.

The function of protein-protein interaction domains in substrate recognition of PTKs

Most of the research on substrate specificity of PTKs has focused on non-catalytic PTK domains or domains found on PTK substrates. For example, Src homology 2 (SH2) domains found on numerous cytosolic signalling

Recognition and specificity in protein tyrosine kinase-mediated signalling

Zhou Songyang and Lewis C. Cantley

There are several factors that contribute to the specificities of protein tyrosine kinases (PTKs) in signal transduction pathways. While protein-protein interaction domains, such as the Src homology (SH2 and SH3) domains, regulate the cellular localization of PTKs and their substrates, the specificities of PTKs are ultimately determined by their catalytic domains. The use of peptide libraries has revealed the substrate specificities of SH2 domains and PTK catalytic domains, and has suggested cross-talk between these domains.

molecules mediate direct interaction with tyrosine-phosphorylated PTKs. The associated proteins can then be phosphorylated for further regulation. Other domains, such as Src homology 3 (SH3), pleckstrin homology (PH) and amino-terminal myristoylation domains, localize PTKs and their substrates to specific compartments of the cell and provide specific contacts with other proteins in signalling cascades^{5,6}. These domains increase the probability that a certain subfraction of potential cellular substrates become phosphorylated.

The SH2 domain is probably the best characterized domain that mediates specificity in PTK signalling. This globular domain of approximately 100 amino

acids has a pocket that directly binds the phosphotyrosine moiety of phosphoproteins or phosphopeptides^{7,8}. Activation of receptor tyrosine kinases [e.g. the platelet-derived growth factor (PDGF) receptor] results in receptor dimerization and transphosphorylation between receptor monomers. This modification creates binding sites for SH2 domain-containing cytosolic proteins, for example, phosphoinositide 3-kinase (PI 3-kinase), phospholipase C_γ, and Grb2. This initiates signalling cascades at the plasma membrane. The cytosolic PTKs (for example, Src) have intrinsic SH2 domains that can serve either in autoregulation or in binding other tyrosine-phosphorylated proteins.

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Critical Review

mRNA Decay in Prokaryotes and Eukaryotes: Different Approaches to a Similar Problem

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Summary

Over the past 15 years considerable progress has been made in understanding the molecular mechanisms of mRNA decay in both prokaryotes and eukaryotes. Interestingly, unlike other important biological reactions such as DNA replication and repair, many features of mRNA decay differ between prokaryotes or eukaryotes. Even when a particular enzyme like poly(A) polymerase has been conserved, polyadenylation of mRNAs in prokaryotes appears to serve a very different function than it does in eukaryotes. Furthermore, while mRNA degrading multiprotein complexes have been identified in both prokaryotes and eukaryotes, their composition and biochemical mechanisms are significantly different. Accordingly, this review seeks to provide a concise comparison of our current knowledge regarding the pathways of mRNA decay in two model organisms, the prokaryote *Escherichia coli* and the eukaryote *Saccharomyces cerevisiae*.

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Keywords Degradosome; endoribonucleases; exoribonucleases; *Escherichia coli*; exosome; polyadenylation; *Saccharomyces cerevisiae*.

INTRODUCTION

In all living organisms, mRNAs serve as transient intermediates in the conversion of the information encoded in genomic DNA into functional proteins. Even though eukaryotic and prokaryotic cells differ considerably in terms of their physical organization (the presence or absence of a nucleus and organelles) and generation times (up to 24 h vs. 30–60 min), they both utilize mRNA decay as one mechanism for controlling gene expression. As such, since the discovery of mRNAs in the early 1960s, considerable effort has been made to determine the pathways by which these molecules are degraded.

Because all mRNA molecules contain identical phosphodiester backbones with the exception of the 5' methyl G cap in eukaryotes, one might assume that eukaryotes and prokaryotes would employ very similar biochemical approaches for their degradation. In fact, some ribonucleases with comparable *in vitro* biochemical properties are found in both types of organisms, but *in vivo* the activities of many of these enzymes differ at the molecular level. More importantly, as outlined below, the mRNA decay pathways in two of the best-studied model systems, the prokaryote, *Escherichia coli* and the eukaryote, *Saccharomyces cerevisiae*, are strikingly different. It should be noted, however, that not all prokaryotes have pathways identical to *E. coli*, nor do all eukaryotes have mechanisms comparable to *S. cerevisiae*. For a more detailed analysis of each system, readers are encouraged to consult more extensive reviews on mRNA decay (1–3).

STRUCTURAL FEATURES THAT AFFECT mRNA DECAY

As summarized in Table 1, there are some important physical differences between the typical yeast and *E. coli* mRNAs. At the 5' terminus in yeast there is the 5' methyl G cap that serves both to protect the mRNA from degradation by 5' → 3' exonucleases and to promote translation initiation. In *E. coli*, the 5' terminus is triphosphorylated, a situation that appears to inhibit the binding of certain endoribonucleases (Table 2). In addition, in *E. coli* the immediate 5' terminus is not involved in the initiation of translation, but rather the existence of a downstream ribosome binding site (RBS) promotes association with the 3' end of the 16S rRNA.

The coupling of transcription and translation in *E. coli* is another factor that is unique in prokaryotes and affects mRNA stability in that failure to translate an mRNA usually results in its rapid degradation (4). The presence of multiple open reading frames (ORFs) in a single polycistronic transcript is a further feature of *E. coli* transcripts that is not found in yeast. Processing of these polycistronic mRNAs

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Table 1
Important features of mRNAs

Property	<i>S. cerevisiae</i>	<i>E. coli</i>
Transcription and translation	Uncoupled, mRNAs contain considerable secondary structure until translation is initiated in the cytoplasm	Coupled, the presence of ribosomes reduces the prevalence of secondary structures within coding regions
Organization	Monocistronic units	Polycistronic and monocistronic units
5' terminus	5' methyl G cap	5' triphosphate terminus
5' untranslated region (UTR)	Does not seem to affect mRNA decay	Presence of secondary structures can either promote or inhibit mRNA decay
Translation initiation	5' cap binding and ribosome scanning for AUG start codon	Ribosome binding sites (complementary to the 3' end of 16S rRNA) close to translation start codon
3' untranslated region (UTR)	Contains one or more polyadenylation signals; transcription termination coupled to polyadenylation	May contain stem-loop structures arising from either REP elements (69) or Rho-independent transcription terminators
3' terminus	Long 55–75 nt unstructured poly(A) tails	With Rho-independent transcription terminators, short single-stranded runs of U residues (1–4 nt)
Polyadenylation	Most transcripts are polyadenylated	Only a small percentage (< 1–2%) of full-length transcripts are polyadenylated

into smaller units generates new 3' termini which may either stabilize or destabilize the processed fragments.

In *E. coli* polyadenylation can occur after Rho-independent transcription terminators but only a small fraction of these mRNAs (< 2%) are polyadenylated at any given time (5). In addition, poly(A) tails act as targeting signals for mRNA decay (6). In contrast, the majority of yeast mRNA's are polyadenylated, which serves to protect them from decay. Furthermore, yeast mRNAs are both inherently more stable [yeast half-lives range from 1–60 min (7); *E. coli* half-lives range from 10 s–20 min (1)], monocistronic, and are degraded primarily after translation has been initiated.

With *E. coli* mRNAs, secondary and tertiary structures in the 5' untranslated region (UTR) have been shown to exert a significant impact on the stability of specific mRNAs. Specific 5' UTRs confer stabilizing properties when spliced onto otherwise short-lived mRNAs (8). On the other hand, the 361 nt 5' UTR of the *rne* (RNase E) mRNA serves as an instability element, helping RNase E autoregulate its own synthesis (9). With yeast mRNAs, only the presence or absence of the 5' cap has to date been shown to affect their decay.

In the 3' UTR regions there are also considerable differences. For example, in *E. coli* transcription termination can occur in the presence of the Rho protein, leaving a 3' terminus that is generally unstructured. However, termination in a Rho-independent fashion, predicted to occur for almost 50% of transcripts that contain ORFs (10) results in the presence of a stem-loop structure that may contain only a very

short single-stranded region at the immediate 3' terminus. As will be discussed later, this type of structure inhibits the binding of 3' → 5' exonucleases. In contrast, with yeast, termination of transcription by RNA polymerase II is coupled with polyadenylation (11). Specifically, cleavage within the 3' UTR by a yet to be characterized endonuclease generates a specific polyadenylation site. Poly(A) polymerase subsequently adds 55–75 A residues resulting in an unstructured 3' terminus (Table 1).

S. CEREVISIAE

(A) Initiation of mRNA Decay

The major pathways for mRNA decay appear to involve an initial deadenylation step (12) catalyzed specifically by two distinct deadenylases (PAN and Ccr4p) (Table 2, Fig. 1). Both of these enzymes contain multiple subunits (13). Based on genetic analysis, these enzymes account for most if not all the observed deadenylation in yeast (13), even though the cell contains a large number of other 3' → 5' exonucleases as will be discussed later. Interestingly, deadenylation is thought to take place during the process of translation while the mRNA is associated with polysomes.

Once deadenylation has removed the majority of the poly(A) tail, the next step is the removal of the 5' methyl G cap by the combined action of Dcp1p and Dcp2p (14, 15) (Table 2, Fig. 1). This process is regulated at multiple levels. In the first place, the decapping machinery needs to displace the

translation initiation factor eIF4F, a multisubunit complex containing eIF4G, eIF4A and eIF4E (16). Furthermore, the efficiency of decapping is regulated in part by the length of the poly(A) tail and the number of poly(A) binding proteins (Pab1p) associated with it (17). In addition, the binding of the Dcp1p/Dcp2p complex is assisted by a group of proteins called Lsm1-7p (18) and Mrt1p (19) (Fig. 1). Decapping is also enhanced by the Edc1p and Edc2p proteins (20).

Evidence has recently been presented that decapping and subsequent 5' → 3' exonucleolytic decay occurs in cytoplasmic processing bodies (called P bodies) (21). Interestingly, while many of the proteins associated with decapping and 5' → 3' exonucleolytic decay were found in the P bodies, components of the deadenylase and the exosome (to be discussed later) were not (21).

Even though yeast has an elaborate system for the deadenylation-dependent removal of the 5' methyl G cap, it has also been demonstrated that decapping can sometimes occur in the absence of deadenylation (22–24). These observations can be a manifestation of what is called nonsense mediated decay (NMD) of mRNAs, a pathway designed to degrade transcripts that contain premature translation stop codons. Nonsense codons closest to the 5' terminus of the mRNA are most efficiently recognized [for a detailed review see González et al. (3)]. This pathway of mRNA decay involves a series of proteins called Upf1p, Upf2p and Upf3p that form a surveillance complex (25). Upf1p has been shown to interact with the termination release factors eRF1 and eRF3 (25). Once this process occurs, the mRNA is rapidly decapped leading to its subsequent degradation. For the NMD system to work efficiently, certain *cis*-acting elements (downstream elements [DSE]) need to be within 150 nucleotides 3' of the stop codon (26). It has recently been shown that there is also a 3' → 5' NMD decay pathway that involves distributive deadenylation and RNA degradation via the exosome (27).

(B) Terminal Steps in mRNA Decay

A major difference between *S. cerevisiae* and *E. coli* is the apparent lack of endonucleases involved in mRNA decay. Thus, once decapping has been completed, degradation proceeds from the 5' end, employing the Xrn1p 5' → 3' exonuclease (Table 2, Fig. 1). Although Xrn1p is inhibited by secondary structures, the fact that it can follow behind a translating ribosome may reduce this potential problem. Alternatively, there are numerous RNA helicases in the cell that can unwind regions of secondary structure. Since it is not clear whether the enzyme can hydrolyze RNA molecules to completion, yeast has an oligoribonuclease (Rex2p, Table 2) that can degrade any small oligonucleotides that might remain.

While a large portion of cytoplasmic mRNA decay is catalyzed by the Xrn1p exonuclease (28), mRNA decay still proceeds in its absence. This observation led to the discovery

of a multiprotein complex called the exosome, which contains at least eleven proteins (29) (Table 2, Fig. 1). Interestingly, six of these proteins are homologues of *E. coli* RNase PH, a phosphorolytic enzyme related to PNPase but which in *E. coli* is primarily involved in tRNA maturation (30). An additional subunit is a homologue of *E. coli* RNase R, a hydrolytic enzyme (Table 2). The exact basis of how the exosome degrades RNA is still not clear. In addition, while a recent genome-wide analysis has suggested that the bulk of mRNA decay in *S. cerevisiae* proceeds in the 3' → 5' direction (31), technical issues have raised questions about the conclusions drawn from this work.

E. COLI

(A) Initiation of mRNA Decay

In contrast to *S. cerevisiae*, the initiation of mRNA decay in *E. coli* is endonucleolytic for the majority of transcripts, employing at least four different endonucleases. RNase E was first discovered based on its role in the maturation of 5S rRNA (32). More importantly inactivation of RNase E leads to the stabilization of specific mRNAs (33). While the exact nature of an RNase E cleavage site is still unclear (34), it has been shown that binding to a 5' monophosphate terminus significantly stimulates enzymatic activity (35) and promotes dimer and tetramer formation (36). In addition, *in vitro*, RNase E prefers a 5' monophosphate terminus to a 5' triphosphate (37).

Furthermore, RNase E access to certain mRNAs may also be controlled in part by the RNA binding protein Hfq, which has been shown to compete with RNase E for the same A/U rich regions, effectively blocking cleavage (38) (Fig. 2B). It has also been suggested that the RraA protein binds directly to RNase E to inhibit its catalytic activity (39). Genomic analysis suggests that RNase E is involved in the decay of a large number of mRNAs (40).

E. coli contains a second endonuclease, called RNase G, that is 34.1% identical to RNase E over the first 488 amino acids of both proteins. Interestingly, RNase G also appears to bind at a 5' PO₄ terminus and is inhibited by a 5' triphosphate (Table 2) (41). Microarray analysis has shown that inactivation of RNase G leads to increased steady-state levels of 11 *E. coli* mRNAs suggesting that this enzyme may initiate the decay of these transcripts (42). More recently, it has been demonstrated that RNase G serves as a backup in both mRNA decay and 5S rRNA maturation when RNase E is inactivated (43) (Fig. 2B).

RNase III (Table 2) is an enzyme that recognizes specific stem-loop structures (44) (Fig. 2B) and is primarily involved in the maturation of rRNA (45). It has also been shown to be involved in the processing of certain polycistronic operons (46). For example, the polycistronic *rpsO pnp* transcript contains an RNase III cleavage site in its intercistronic region (46). Cleavage at this site leads to a 5–7-fold decrease in the half-life of the *pnp* mRNA (46). However, since the enzyme is

Table 2
Comparison of *E. coli* and *S. cerevisiae* proteins involved in mRNA decay

Enzyme/protein	<i>E. coli</i>	<i>S. cerevisiae</i>
Endoribonucleases	<p>RNase E – 5' end-dependent; inhibited by 5' triphosphate; primary endonuclease in mRNA decay; essential for cell viability</p> <p>RNase G – 5' end-dependent; inhibited by 5' triphosphate: back up enzyme in mRNA decay</p> <p>RNase III – recognizes specific stem-loop structures; cleaves near the base of the stem; cleaves polycistronic mRNAs into smaller units</p> <p>RNase P – contains protein subunit and catalytic RNA subunit; required to generate mature 5' ends of tRNAs; cleaves polycistronic mRNAs into smaller units; essential for cell viability</p> <p>No homolog</p>	<p>No homolog</p> <p>No homolog</p> <p>Rnt1p – localized in nucleus; involved in degradation of unspliced pre-mRNAs and lariat introns; probably not involved in mRNA decay</p> <p>RNase P – contains protein subunit and catalytic RNA subunit; required to generate mature 5' ends of tRNAs; probably not involved in mRNA decay</p>
Exonucleases (5' → 3')	<p>No homolog</p>	<p>Xrn1p – cytoplasmic; releases mononucleotides; inhibited by secondary structures; primary enzyme for degrading cytoplasmic mRNAs</p> <p>Rat1p – nuclear; releases mononucleotides; inhibited by secondary structures</p> <p>No homolog</p>
Exonucleases (3' → 5')	<p>PNPase – phosphorolytically produces nucleoside diphosphates; reversible <i>in vivo</i>; inhibited by secondary structures; does not degrade short oligonucleotides; primary exonuclease involved in mRNA decay</p> <p>RNase II – hydrolytically produces nucleoside monophosphates; inhibited by secondary structures; does not degrade short oligoribonucleotides</p> <p>RNase R – homolog of RNase II; hydrolytic; not inhibited by secondary structure; involved in rRNA processing and degradation; probably involved in mRNA decay</p> <p>RNase PH – homolog of PNPase that phosphorolytically produces nucleoside diphosphates; involved in tRNA maturation but not mRNA decay</p>	<p>No homolog</p> <p>Rrp44p/Dis3p – essential exosome component</p> <p>Rrp41/Ski6p – essential exosome component</p> <p>Rrp42p – essential exosome component</p> <p>Rrp43p – essential exosome component</p> <p>Rrp45p – essential exosome component</p> <p>Rrp46p – essential exosome component</p> <p>Mtr3p – essential exosome component</p> <p>Rex2p – specific for short oligonucleotides</p>
Oligoribonuclease	<p>Oligoribonuclease – specific for short oligoribonucleotides; essential for cell viability</p>	

(continued overleaf)

Table 2
(continued)

Enzyme/protein	<i>E. coli</i>	<i>S. cerevisiae</i>
RNA helicases	RhlB—when associated with RNase E hydrolyzes ATP and unwinds RNA	Ski2p—putative RNA helicase associated with exosome
Poly(A) binding proteins	CspE—cold shock protein; binds to poly(A) <i>in vitro</i> Ribosomal protein S1—required for protein synthesis; binds to poly(A) <i>in vitro</i>	No homolog Rrp4p—essential exosome component
	No homolog	Rrp40p—essential exosome component Csl4p—essential exosome component PABp—promotes stability of poly(A) tails and stimulates translation through interaction with initiation complex
Poly(A) nucleases	PNPase—degrades poly(A) tails preferentially associated with mRNAs RNase II—degrades poly(A) tails preferentially associated with rRNA No homolog No homolog None have been identified	No homolog No homolog PAN—specifically degrades poly(A) tails Ccr4p—specifically degrades poly(A) tails Dcp1p/Dcp2p No homolog
Decapping enzymes	Hfq—binds to A/U rich regions, some of which are RNase E cleavage sites	Lsm1-7p—seven proteins that are associated with Dcp1p and Dcp2p and are required for decapping
Auxiliary proteins	No homologs No homologs	Ski7p—putative GTPase associated with the exosome Ski2p/Ski8p—proteins associated with the exosome

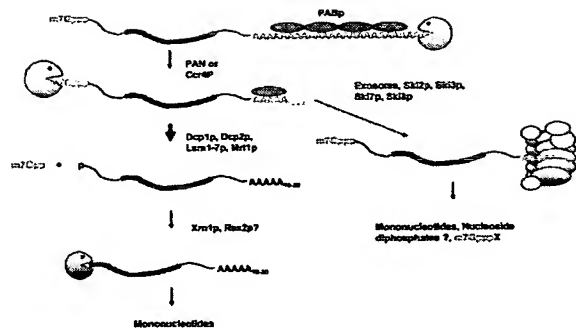


Figure 1. mRNA decay pathways in *S. cerevisiae*. The typical yeast mRNA will have a 5' methyl G cap and a poly(A) tail of 55–75 nt. Deadenylation by either the PAN or Ccr4P poly(A) nuclease displaces bound poly(A) binding proteins (PABp) and initiates decay. Decapping by a complex of proteins including Dcp1p/Dcp2p (the actual decapping enzyme), Lsm1-7p and Mrt1p displaces translation initiation factors (not shown for sake of simplicity) from the cap structure once the poly(A) tails have been shortened to the 10–20 nt range. Once decapping has occurred, the 5' → 3' Xrn1p exonuclease processively degrades the mRNA. Any small oligoribonucleotides remaining if Xrn1p to degrade is unable to degrade to completion would be substrates for oligoribonuclease (Rex2p). Alternatively, following deadenylation some mRNAs may become substrates for the exosome complex (11 proteins, see Table 2) along with Ski2p, Ski3p, Ski7p and Ski8p. Ski7p is a putative GTPase while Ski2p has been shown to be an RNA helicase. The exosome is depicted as a large multiprotein complex with its associated Ski proteins. The dotted arrow indicates that the 3' → 5' exosome pathway is less frequently employed than 5' → 3' Xrn1p mediated degradation. Since the majority of the exosome subunits are homologs of the phosphorolytic enzyme RNase PH, it would be assumed that nucleoside diphosphates would be produced by exosome cleavage. This has not yet been confirmed. Drawing not to scale.

not essential for cell viability (47), it has been assumed that RNase III does not play a major role in initiating mRNA decay. RNase P (Table 2), the enzyme required for generating the mature 5' ends of tRNAs (48) has also been recently shown to cleave a limited number of polycistronic transcripts, including the *hisL*, *rbsD* and *tnaL* operons, within their intercistronic spacer regions (49). There does not appear to be any overlap between RNase III and RNase P cleavage sites.

The inhibition of RNase E and RNase G activity by 5' triphosphates raises the interesting question regarding whether this sequence might function as a prokaryotic specific cap. For polycistronic mRNAs, cleavages within intracistronic spacer regions by RNase III or RNase P partially solves this problem,

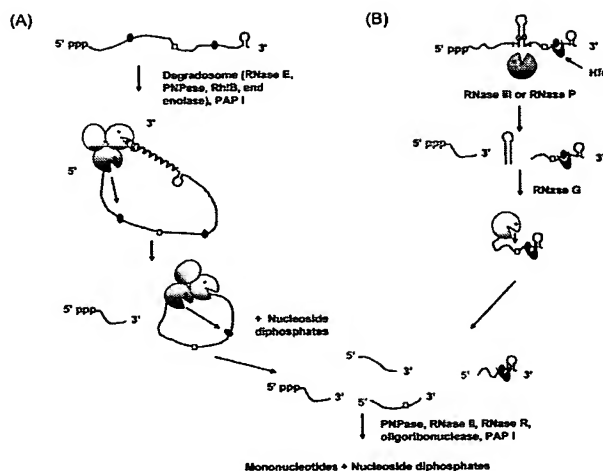


Figure 2. mRNA decay pathways in *E. coli*. (A) mRNA decay catalyzed by the RNase E based degradosome. The degradosome contains RNase E, PNPase, the RhlB RNA helicase and the glycolytic enzyme enolase. For the sake of simplicity the enolase subunit has been omitted. In the example shown here a monocistronic mRNA terminated with a Rho-independent transcription terminator (stem-loop structure) is shown. Prior to the initiation of decay, the 3' end of the transcript is polyadenylated by PAP I (average tail length is 10–45 nt). The PNPase protein would bind to the poly(A) tail while RNase E would bind (inefficiently) to the 5' triphosphate terminus or perhaps at an internal site. The order of binding has not been established at this point. The first endonucleolytic cleavage by RNase E would generate a 5' PO₄ terminus which would stimulate the activity of RNase E. While this was occurring PNPase would be degrading exonucleolytically from the 3' terminus releasing nucleoside monophosphates. The oligonucleotides generated by RNase E cleavage would subsequently be degraded by a combination of PNPase, RNase II and RNase R. Fragments containing secondary structures would be polyadenylated to facilitate their degradation. The terminal degradation products of PNPase, RNase II and RNase R (short oligonucleotides, 4–7 mers) would be degraded by oligoribonuclease. (B) Degradation of a polycistronic mRNA. In the example shown, RNase III cleaves on both sides of a stem-loop structure within the intercistronic region (marked by vertical black lines). The downstream gene contains both an RNase G and RNase E cleavage site. In this case the Hfq protein is bound to this A/U rich region so RNase G cleaves at its site which is not protected. Endonucleolytic cleavage products are degraded by either PNPase, RNase II or RNase R. In cases where there is a stem-loop structure, polyadenylation takes place. For a small percentage of *E. coli* transcripts, the entire decay process proceeds exonucleolytically. Small oligonucleotides are degraded by oligoribonuclease. Drawing not to scale.

since the downstream cleavage products will have a 5' phosphomonoester terminus (Fig. 2B). For monocistronic transcripts, binding to the 5' triphosphate may be the rate-limiting step in the initiation of mRNA decay (Fig. 2A). Recently, however, it has been shown that RNase E can initiate cleavages at internal entry sites (50) (Fig. 2).

Another interesting aspect of mRNA decay in *E. coli* is the function of the multiprotein complex called the degradosome. As initially identified via copurification and immunoprecipitation experiments, the degradosome has been shown to contain RNase E, PNPase, the RhlB RNA helicase and the glycolytic enzyme enolase (51, 52). More recently evidence has been presented that this multiprotein complex is possibly associated with the inner membrane of the bacterium (53). Because this complex contains both endo- and exonucleolytic activity as well as a helicase that can unwind secondary structures, it has been generally assumed that it is responsible for the majority of mRNA decay in *E. coli* (Fig. 2A).

It is not clear whether the degradosome binds initially to the 3' terminus via PNPase or to the 5' terminus via RNase E (Fig. 2A). However, experiments with RNase E mutants in which the degradosome cannot be assembled, have shown that mRNA decay is normal under these circumstances (54). Thus, the nucleolytic activities of RNase E and PNPase are independent of their physical interaction. In addition, the degradosome scaffolding region of the *E. coli* RNase E is only conserved in a limited number of bacteria. However, recent experiments suggest that the degradosome may play a role in the degradation of mRNA fragments that contain REP (repetitive extragenic palindromes) elements (55).

One of the hallmarks of bacteria is their ability to rapidly react to significant changes in their growth conditions. As such, under conditions of stress, besides the normal pathways for degrading mRNAs, *E. coli* has at least one additional mechanism for the rapid functional inactivation of mRNAs. In particular, the RelE protein, a bacterial toxin that is induced under stress conditions (56), has now been shown to stimulate the cleavage of mRNAs in the ribosomal A site (57), thereby rapidly inhibiting translation. Once the cleaved mRNA is released from the ribosome, possibly by the intervention of tmRNA mediated trans-translation system (58), further steps in its degradation would proceed as described in the next section.

Finally, it should be noted that certain data suggest that some mRNAs may be decayed in a strictly 3' → 5' exonucleolytic fashion (see next section for more details) (Fig. 2). Thus the half-lives of some mRNAs are unaffected in RNase E RNase G double mutants (43). In addition, recent genome-wide analysis has shown that inactivation of PNPase leads to the stabilization of close to 17% of transcribed ORFs (59). Interestingly, it was also shown that the presence of RNase II, the major 3' → 5' exonucleolytic activity in *E. coli*, actually stabilized a significant fraction of *E. coli* transcripts (59).

Accordingly, *E. coli* employs polyadenylation to facilitate the action of both PNPase and RNase II. The polyadenylation process has been shown to stimulate the degradation of both full-length and partially degraded mRNAs (5, 6). In fact, it has been argued that the rapid turnover of polyadenylated mRNA species in part accounts for their low abundance (5).

(B) Terminal Steps in mRNA Decay

Since *E. coli* does not contain any 5' → 3' exonucleases, all exonucleolytic degradation of decay intermediates or full-length mRNAs proceeds in the 3' → 5' direction. Furthermore, although *E. coli* has at least seven 3' → 5' exonucleases (60), only three of them (PNPase, RNase II, oligoribonuclease, Table 2) have been genetically implicated in mRNA decay (61, 62). However, a role for RNase R, a homologue of RNase II (Table 2), in the degradation of mRNA fragments cannot be ruled out. It is important to note that both PNPase and RNase II are inhibited by secondary structures (63) and do not bind efficiently to short single-stranded regions (63). Furthermore, since neither PNPase nor RNase II can degrade an RNA substrate to completion, oligoribonuclease hydrolyzes the small oligoribonucleotides (4–7 nt) that remain (62) (Table 2, Fig. 2). In fact, this step has been shown to be essential for cell viability (62).

Even though the bulk of the exonucleolytic activity observed in crude lysates of *E. coli* is accounted for by RNase II (64), it now appears the PNPase plays a more significant role in mRNA decay (59). Degradation by PNPase may be further stimulated by its interaction with the RhlB RNA helicase (65), an enzyme that unwinds RNA particularly when it is associated with the RNase E based degradosome (66) and possibly in a complex with PNPase (65).

CONCLUSIONS

In yeast the presence of poly(A) tails and their associated poly(A) binding proteins serve to protect them from degradation. Once deadenylation is initiated, removal of the 5' methyl G cap leads to rapid exonucleolytic degradation from either the 5' or 3' termini (Fig. 1). While 5' → 3' degradation is carried out by a single enzyme (Xrn1p), decay from the 3' terminus involves a multiprotein complex (exosome) that contains numerous putative 3' → 5' exonucleases. At this time an explanation for this difference in approach is not particularly obvious. In addition, further work is necessary to establish whether only 5' → 3' decay takes place within the P bodies and to determine the relationship the normal mRNA decay pathways relative to nonsense mediated decay.

In contrast, with *E. coli* it seems probable that additional endoribonucleases will be identified, since inactivation of RNase E, RNase G, RNase P, and RNase III does not completely block the initiation of mRNA decay. In addition, there also appears to be a pathway of mRNA decay that involves the interaction of small regulatory RNAs with their

target mRNAs (67). Finally, it is not understood why the failure to degrade decay intermediates in PNPase RNase II double mutants (61) or oligoribonuclease deficient strains (62) leads to a loss of cell viability.

An interesting aspect of mRNA decay in both organisms is the presence of multiprotein complexes that are involved in mRNA decay. In the case of the yeast exosome, all eleven proteins are essential components for enzymatic activity. It thus takes eleven proteins to serve as a single 3' → 5' exonuclease, although it is not clear whether the complex has both phosphorolytic and hydrolytic activity. In contrast, *E. coli* contains the degradosome, composed of RNase E, PNPase, the RhlB RNA helicase and enolase. Both complexes can degrade mRNAs, but the degradosome is multifunctional compared to the exosome in that it contains both endo- and exonucleolytic activity.

Taken together it is apparent that yeast and *E. coli* have taken significantly different routes to deal with a common problem. Some of the variations may simply relate to the inherent structural differences in the mRNAs, in particular the presence of the 5' methyl G cap. However, it is not clear why *E. coli* lacks a 5' → 3' exonuclease and *S. cerevisiae* does not employ endonucleases. Thus, the lack of any 5' → 3' exonucleases in *E. coli* necessitates an alternative approach to degrading transcripts, although the 5' end dependence of RNase E and RNase G does provide the cell with the ability to degrade transcripts from the 5' terminus. Furthermore, the two organisms employ polyadenylation very differently.

Finally, it is interesting to note that degradation from the 5' terminus would seem to be the most efficient form of degradation, in terms of the rapid functional inactivation of mRNAs. However, in *E. coli* and to a very limited extent in yeast many mRNAs are degraded primarily from their 3' termini, suggesting the possibility of significant levels of truncated proteins that arise as a result of partial mRNA decay. At least in *E. coli* these mRNAs and their encoded proteins appear to be targeted for degradation by the translation system associated with the *ssrA* encoded tmRNA (58, 68). Clearly additional research will be necessary to gain a more complete understanding of the pathways of mRNA decay in both organisms.

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hydrogen transfer reactions need to be studied, in order to test the generality of this new phenomenon. It would also be of considerable interest to know if particles heavier than protium and deuterium display quantum mechanical behavior in enzyme reactions at room temperature. With regard to modeling, this represents an extremely important aspect of the problem. It is expected that successful fitting of experimental data will provide unique insights into the shape of reaction coordinates, as well as the role of protein dynamics, in enzyme catalysis.

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Poly(A), poly(A) binding protein and the regulation of mRNA stability

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Gene expression can be controlled by changing the half-lives of specific mRNAs in response to intracellular events or to extracellular stimuli. Many experiments suggest that mRNA turnover in mammalian cells is linked to poly(A) metabolism, which might be affected by the efficiency of the cytoplasmic poly(A) binding protein in protecting poly(A) from nucleolytic attack.

Most newly synthesized mRNAs are modified post-transcriptionally by the addition of a poly(A) tract at their 3' termini. Although the detailed mechanisms of poly(A) synthesis are being elucidated, the functions of poly(A) are not fully understood. The poly(A) tract might have multiple functions, both in the nucleus and the cytoplasm: it might play a role in the processing and transport of mRNA (for review see

Ref. 1); it affects the translational efficiency of mRNAs, particularly in lower eukaryotes²; and it seems to influence mRNA turnover. This review will focus on the potential role of poly(A) and its associated poly(A) binding protein (PABP) in determining the half-lives of mRNAs.

Poly(A) can influence mRNA stability

Most of the data regarding poly(A) and mRNA metabolism suggests that poly(A) can protect mRNA from rapid destruction. Some initial evidence came from experiments showing the time-dependent shortening of poly(A) tracts. For example, the poly(A) tracts

of newly synthesized globin mRNA molecules in splenic erythroid cells contain approximately 150 adenylate (A) residues, which are progressively shortened to 40–60 As (Ref. 3). This and other experiments were not designed to show a direct cause and effect relationship between poly(A) shortening and mRNA turnover, but they did indicate a strong correlation between the two.

In subsequent experiments the half-lives of an mRNA with or without its poly(A) tract were compared directly. Rabbit globin mRNAs containing poly(A) tracts of varying lengths were co-injected with radiolabeled histidine into *Xenopus* oocytes⁴. The functional stability (ability to be translated) of each mRNA preparation was then determined by measuring the time span over which globin protein was synthesized. In this assay mRNA molecules with poly(A) tracts containing 32 or more A residues have the same functional stability as mRNAs with 150 As. In contrast, the stability of the same mRNA with only 16 As is at least ten-fold lower and is close to that of fully deadenylated mRNA. Nudel and colleagues reasoned that the functional stability of each mRNA is directly related to its structural stability, since the translational efficiency of deadenylated mRNA is the same as that of its

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polyadenylated counterpart during the first few hours after injection.

Further support for the poly(A) protection idea came from microinjection and *in vitro* mRNA decay experiments. The functional stability of histone mRNA, which normally lacks poly(A), is enhanced in oocytes if the mRNA is polyadenylated prior to injection⁵. Similarly, using an *in vitro* mRNA decay system (which will be described below), the half-lives of polyadenylated globin and histone mRNAs are at least tenfold longer than that of their deadenylated counterparts⁶. The major conclusion from these experiments is that the stability of at least some mRNAs is influenced by whether or not they are polyadenylated.

Poly(A) shortening and mRNA degradation

If poly(A) tracts serve to protect some mRNAs from nuclease attack, then poly(A) metabolism is probably linked with mRNA turnover. In other words, an mRNA might remain intact as long as its poly(A) is maintained at some minimal length. In order to demonstrate a direct link between the rate of poly(A) removal and mRNA turnover, it is necessary to show evidence for a precursor-product relationship between poly(A) loss and mRNA destruction, and we review that evidence below.

The poly(A) tract of newly synthesized metallothionein mRNA contains approximately 150–200 residues, which shortens to approximately 30 residues over a period of 5 to 10 hours⁷. During the next several hours, the steady-state level of the mRNA decreases, but fully deadenylated mRNA is not observed. The simplest interpretation of these results is that the body of the mRNA is

protected from degradative nucleases while its poly(A) tract contains at least 30 to 40 residues. When more As are removed, the mRNA body is protected less efficiently and is rapidly destroyed, accounting for the absence of a pool of deadenylated metallothionein mRNA. Analogous results have been observed for other mRNAs, as follows:

- (1) Variations in tubulin mRNA levels occur throughout the cell cycle of the *Physarum polycephalum* amoeba and result, in part, from changes in tubulin mRNA stability⁸. These changes, in turn, correlate directly with poly(A) length. When tubulin mRNA is scarce (after mitosis), its poly(A) tract is relatively short (~30 As). During G₂, as the tubulin mRNA level begins to rise, its poly(A) tract is longer (~80 As).
- (2) In exponentially growing human myeloid cells, *c-myc* mRNA degradation seems to follow a sequential pathway, the first step of which is poly(A) removal⁹.
- (3) The first steps of *c-fos* mRNA decay in HeLa cells also involve poly(A) shortening and removal¹⁰.

To our knowledge, there is no way to inhibit poly(A) shortening specifically in cells. Such a method would permit a direct test of the link between poly(A) shortening and mRNA degradation.

In any event, additional evidence supporting that link is provided by experiments using a cell-free mRNA decay system. Before describing the data, we shall review briefly several properties of the system developed in our laboratory; similar systems have been described by other investigators^{11–13}. The *in vitro* reactions include polysomes prepared from K562 cells, a human erythroleukemia line¹⁴. The decay rates of endogenous, polysome-bound mRNAs are monitored by incubating the polysomes under appropriate conditions, extracting RNA, and then hybridizing it with radiolabeled probes. The mRNAs are degraded at different rates by an undetermined number of nucleases that sediment with the polysomes. The decay of radiolabeled mRNA substrates can also be assayed in this system, using either polysomes or a high-salt extract of the polysomes as a source for messenger ribonucleases. The high-salt treatment solubilizes most or all of these nucleases.

There are several advantages of using *in vitro* systems for studying the process of mRNA decay.

- (1) Transcriptional inhibitors, which are rarely, if ever, specific for transcription alone, are unnecessary.
- (2) Short lived mRNA decay products that are difficult to detect in cells can sometimes be observed *in vitro*, where reaction rates are considerably slower.
- (3) It becomes feasible biochemically to analyse the effects of cellular components which might regulate mRNA stability. We and others have discussed several properties of the *in vitro* systems which indicate that they can accurately reflect mRNA turnover processes in whole cells^{11–14}. The most important property is that the rank order of decay of various polysome-associated mRNAs *in vitro* reflects that observed in intact cells.

In the *in vitro* system we have described, poly(A) removal precedes the degradation of *c-myc* mRNA¹⁵ (Fig. 1). Polysomes were incubated in standard reaction mixtures, RNA was isolated, and the 3' region of *c-myc* mRNA was analysed by an RNase H-mapping technique. The data indicate that *c-myc* mRNA decay *in vitro* is a stepwise process, in which shortening of the poly(A) tract precedes nuclease attack within the 3'-untranslated region. In contrast, the poly(A) tract of long lived β -globin mRNA remains stable throughout the incubation. In this experiment, therefore, as in those with intact cells, poly(A) removal correlates with mRNA stability.

Poly(A) elongation and mRNA stabilization

We have discussed two groups of observations that suggest a link between poly(A) metabolism and mRNA half-lives. (1) Poly(A) removal seems to precede the degradation of some mRNAs. (2) The stability of non-polyadenylated mRNAs is enhanced by adding poly(A) tracts to their 3' termini. Additional evidence supporting the poly(A) protection hypothesis comes from the observation that the stabilization and/or accumulation of several mRNAs is accompanied by elongation of their poly(A) tracts.

Paek and Axel¹⁶ analysed the expression in mouse fibroblasts of a series of human growth hormone (hGH) constructs driven by the herpes simplex virus thymidine kinase promoter. When the cells are exposed to glucocorticoids, hGH expression increases, in part because the hGH mRNA is stabilized. As determined by northern blot analysis, the size of hGH

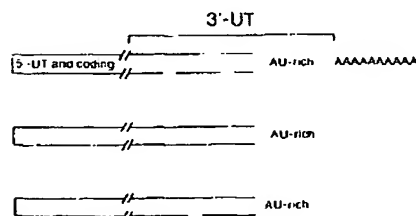


Fig. 1. Initial steps in the pathway of *c-myc* mRNA degradation *in vitro*. The first detectable step in the *in vitro* pathway is the shortening of the poly(A) tract. As the incubation continues, all of the poly(A) is removed, and soon thereafter several additional mRNA decay products appear. The 3' termini of these products are located within the AU-rich 3'-untranslated (3'-UT) region.

mRNA also increases by approximately 100 nucleotides in glucocorticoid-treated cells. However, the size of the mRNA body does not change, because hGH mRNAs extracted from uninduced and induced cells are identical in size if they are first hybridized to oligo(dT) and treated with RNase H [to remove their poly(A) tracts]. Therefore, the glucocorticoid-induced elongation of hGH mRNA results from the addition of A residues to the poly(A) tract and occurs during the same time period that the mRNA is stabilized. One explanation for these results is that the stabilization of hGH mRNA correlates with lengthening of its poly(A) tract.

Recent experiments suggest a similar correlation between poly(A) elongation and the accumulation of vasopressin mRNA in the rat hypothalamus. When rats are subjected to osmotic stress, vasopressin mRNA accumulates to higher than normal levels, and its associated poly(A) tract is lengthened by approximately 150 residues¹⁷. Normal circadian variations in vasopressin mRNA levels also correlate with changes in poly(A) tract size¹⁸. While the mechanism for these fluctuations has not been defined, it is possible that the mRNA is stabilized as it accumulates and that poly(A) elongation plays a role in prolonging its half-life. Similar links between poly(A) length and changes in the stability and translatability of 'maternal' mRNAs has been observed following fertilization and during early embryogenesis¹⁹.

Poly(A)-protein interactions

Several observations imply that, once a poly(A) tract has been shortened to less than some critical length (30–40 residues), the remainder of its As are rapidly excised, generating a deadenylated mRNA which can be more susceptible (relative to its polyadenylated counterpart) to nuclease attack. In oocytes, an mRNA with a poly(A) tract of less than 32 residues has approximately the same half-life as its deadenylated counterpart⁴. When *c-myc* mRNA is being degraded *in vitro* or when metallothionein mRNA is being degraded in cells, their poly(A) tracts are shortened until 30 to 40 A residues remain^{7,15}. mRNAs with shorter poly(A) tracts are not observed. Taken together, these data suggest that poly(A) shortening occurs very rapidly once the poly(A) tract has reached some minimum length.

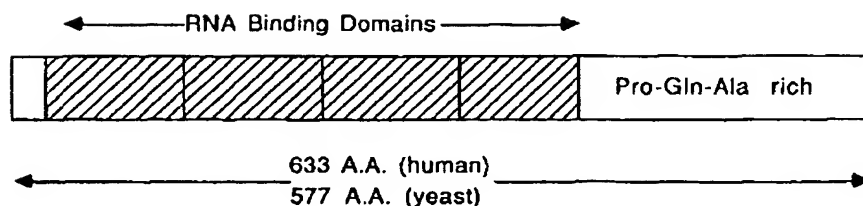


Fig. 2. Schematic representation of poly(A) binding protein. The four RNA binding domains of the N-terminal region are indicated as striped boxes. Each of the domains is homologous to the others, and to RNA binding domains of other nucleic acid binding proteins. The region indicated as proline-rich is not conserved between the human and yeast proteins and is notably depleted of acidic amino acids in the human protein. Thus, the scheme is representative of the human version. The function of this C-terminal domain is unknown.

This observation can be explained by the fact that poly(A) is organized into a nucleosome-like ribonucleoprotein, by virtue of its association with poly(A) binding protein (PABP). This protein, discovered over 15 years ago²⁰, binds with high specificity to poly(A) and is complexed to cytoplasmic mRNAs. When cytoplasmic messenger ribonucleoproteins (mRNPs) are digested with ribonuclease T2, fragments of poly(A) containing approximately 27 residues and multiples thereof are generated. In contrast, when deproteinized poly(A)-containing mRNA is digested with the same RNase, random digestion products are generated, indicating that the protein portion of the RNP is responsible for the 27 residue repeat structure. Subsequently, it was possible to reconstitute the same sort of RNP structure simply by mixing poly(A) with purified PABP²¹. Therefore, a single PABP molecule is thought to bind up approximately 27 As and, thereby, to protect them from nuclease attack. The mRNA stability experiments discussed above are thus consistent with a model whereby, once a poly(A) tract has been shortened to less than about 27 As, it can no longer interact with a PABP molecule and becomes an easy target for nuclease digestion.

Significant progress in our understanding of the structure and function of PABP was made when several groups succeeded in cloning PABP sequences from humans and from the yeast, *Saccharomyces cerevisiae*^{22–24}. The overall structure of the protein (577 amino acids in yeast, 633 amino acids in humans) is conserved from yeast to humans and consists of two major domains. The N-terminal domain is highly conserved and contains four segments with short sequences that are homologous both to each other and to segments of other RNA binding proteins (see Fig. 2).

This four-segment domain is probably required for the interaction of the protein with poly(A), because only one of the four RNA binding segments of yeast PABP is sufficient for binding poly(A) and for maintaining cell viability²⁵. The N-terminal domain also seems to be responsible for the capacity of PABP to migrate from one poly(A) molecule to another. This property might play an important role in its effect on mRNA stability (see below). The C-terminal domain is less well conserved and is rich in proline, glutamine and alanine. Its function is unknown, but it might interact with cytoplasmic factors that regulate mRNA stability. Deletion of *PAB* (the PABP gene) from yeast is lethal showing that PABP is an essential protein²⁵. These data indicate that, whatever its role(s) in the cell, poly(A) does not exist as free RNA but is bound up with PABP. It is not known whether other proteins also bind to poly(A) tracts.

Poly(A)-PABP complex and mRNA stability

Many investigators have suggested that poly(A), in association with PABP, functions to protect mRNAs from rapid attack by cellular RNases. Additional support for this idea came from the observation that the poly(A) tract of mRNP is considerably more resistant to digestion *in vitro* by snake venom exonuclease than is the poly(A) of protein-free mRNA²⁶.

Recent approaches have permitted more direct tests of the poly(A)-PABP stabilization hypothesis. We investigated whether the long half-life of β -globin mRNA was dependent on PABP in our cell-free mRNA decay system²⁷. Reactions were prepared containing a radiolabeled, polyadenylated β -globin mRNA substrate, ribosomal salt wash (the source of PABP and of mRNA-degrading nucleases), and different amounts of unlabeled

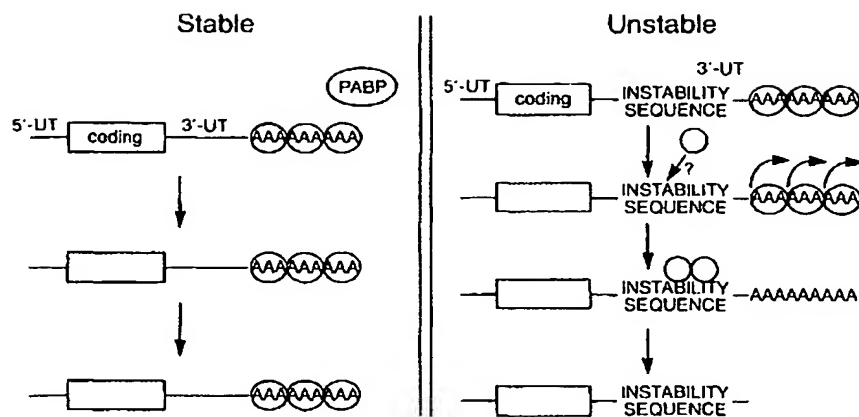


Fig. 3. Proposed model linking poly(A) and PABP with mRNA stability. Specific sequences can confer stability upon their associated polyadenylated mRNAs. These sequences (indicated as 'instability sequences') might act either independently or in concert with other factors (tinted circles) to destabilize the poly(A)-PABP complex. The PABP-depleted poly(A) would be susceptible to nucleolytic attack. The more stable mRNAs lack instability sequences and thus maintain their poly(A)-PABP complexes, which makes these mRNAs relatively resistant to nucleases.

competitor poly(A). In the absence of competitor poly(A) or in the presence of poly(G), poly(U), or poly(C), the mRNA is quite stable. The addition of competitor poly(A) leads to a seven-fold increase in β -globin mRNA decay. None of the four ribohomopolymers affects the decay of non-polyadenylated histone mRNA. One explanation for these results is that PABP, which is originally bound to the β -globin substrate, migrates away from the mRNA and onto the competitor poly(A), presumably by virtue of the multiple RNA binding sites. As a result, the poly(A) tract of the mRNA retains little or no bound PABP, and the mRNA is destabilized.

To test this hypothesis more directly, the decay rate of β -globin mRNA was compared in reactions containing either unmodified ribosomal salt wash (which contains PABP) or salt wash that had been depleted of approximately 90% of its PABP by chromatography through a poly(A)-Sepharose column. The mRNA is degraded approximately seven times faster in reactions deficient in PABP than it is with PABP, and degradation follows a sequential pathway, in which rapid poly(A) removal precedes digestion of the mRNA body. When purified PABP is added back to the PABP-deficient salt wash, the mRNA is stabilized, and its poly(A) tract is shortened more slowly. PABP has no effect on the decay of non-polyadenylated histone mRNA or on deadenylated β -globin mRNA. These results support the idea that a poly(A)-PABP complex is

necessary to maintain the long half-life of this mRNA, at least *in vitro*.

Differential mRNA stability: a hypothesis

Many experiments with intact cells, as well as with some *in vitro* systems, suggest that poly(A) removal precedes the degradation of the mRNA body. *In vitro* experiments support the idea that PABP can protect the poly(A) tract (and the rest of the mRNA as well) from nuclease attack. If PABP serves that function in cells, it is necessary to account for differences in mRNA half-lives in terms of differential poly(A) shortening rates. However, if the poly(A) tract of every mRNA is associated with PABP, how can the poly(A) shortening rate vary from one mRNA to another?

One clue to this apparent paradox comes from mapping studies showing that mRNA sequences that affect stability can also affect poly(A) shortening. For example, the stable mRNA for β -globin was modified by deleting its 3'-untranslated region and replacing it with a portion of the 3'-untranslated region from the unstable mRNA for granulocyte, macrophage colony stimulating factor (GM-CSF). The stability of the hybrid transcript *in vivo* is similar to that of unmodified GM-CSF mRNA²⁸. Likewise, *c-fos* mRNA with deletions in its 3'-untranslated region is more stable than its unmodified counterpart¹⁰. The 3'-untranslated regions of *c-fos*, GM-CSF and some other unstable mRNAs are particularly rich in A and U residues²⁹, and the same

AU-rich mRNAs undergo rapid poly(A) shortening. The poly(A) tract of *c-fos* mRNA has a short half-life in cells, but only if the AU-rich portion of the 3'-untranslated region is present¹⁰.

These and other observations indicate that specific regions of an mRNA can influence its half-life. Some of these regions are AU-rich, while others, like the coding region of *c-fos* mRNA, are not³⁰. These same observations also provide the basis for a model whereby poly(A) shortening and the stability of the poly(A)-PABP complex are determined by specific mRNA sequences (Fig. 3). The model requires that these so-called instability sequences, either by themselves or in concert with cellular factors, increase the susceptibility of the poly(A) tract to nuclease attack. The hypothesis is that the sequences function in some way by affecting the association of PABP with the poly(A) tract. That is, instability sequences might interfere with the capacity of PABP to protect the poly(A) tract. The mechanism of this interference is obscure but might depend upon the capacity of the protein to migrate from one site to another. An important, but untested, prediction of this model is that the affinity of PABP for the poly(A) tracts of stable and unstable mRNAs differs.

Summary

This review has focused on the possibility that interactions between mRNA sequences and the poly(A)-nucleoprotein complex play important roles in mRNA turnover. It is important to stress that additional genetic and biochemical tests are necessary to characterize how PABP interacts with mRNA in cells and to determine whether the poly(A) protection hypothesis is accurate. Moreover, there may be a significant number of mRNAs whose half-lives are independent of polyadenylation. For example, the stabilities of poly(A)-containing and deadenylated α_2 -globulin and interferon mRNAs are similar in microinjected oocytes^{31,32}. Thus, an important challenge in this field will be to analyse the complex and interactive factors that determine the half-lives of specific mRNAs.

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Coordinated regulation of morphological and biochemical differentiation in a steroidogenic cell: the granulosa cell model

Abraham Amsterdam, Siegfried Rotmensch and Avri Ben-Ze'ev

Studies on the dynamic biochemical and morphological events occurring during steroidogenesis in granulosa cells suggest that the organization and expression of the actin-cytoskeleton may play a major role in the transduction of endocrine and paracrine steroidogenic signals, and in the coordination between the organelles involved in this process. Since steroid hormones are not stored intracellularly, regulation of their production is dependent mainly on the expression of genes coding for membrane-bound steroidogenic enzymes. Recently, the expression of oncogenes of the ras family was also implicated in the regulation of steroidogenesis.

Differentiation of granulosa cells (GCs) within an oocyte-enclosed ovarian follicle is a prerequisite for ovulation in mammals. The maintenance of early pregnancy depends on the transformation of GCs (Fig. 1) into highly differentiated lutein cells which comprise the main source for the secretion of the steroid hormone progesterone (for reviews see Refs 1–3).

The development of the steroidogenic potential in differentiating GCs

consists of early and late events. The former includes mobilization of cholesterol, reorganization of endosomes, lysosomes, lipid droplets and particularly mitochondria and smooth endoplasmic reticulum (SER), which are the carriers of membrane-bound enzymes converting cholesterol to a variety of steroid hormones (Fig. 2, and Ref. 3). The late events consist of stable changes in cell shape, intercellular communication through gap junction formation and the development of organelles associated with steroidogenesis (Fig. 3, and Ref. 3). *De novo* synthesis of membrane-bound steroidogenic enzymes correlates at the ultrastructural level with morphological changes in mitochondria and SER³. Stimulated steroidogenesis during GC

differentiation can be evoked by a variety of endocrine and paracrine factors interacting with specific receptors^{1–3}, and it raises several intriguing questions:

- (1) What are the biochemical signals generated at the cell membrane to initiate steroidogenesis?
- (2) How do these signals reach the intracellular steroidogenic machinery?
- (3) How do the signals, generated by various stimulants, 'cross-talk' in order to arrive at a well coordinated message?
- (4) Which genes are expressed during the modulation of steroidogenic activity?

The studies described below relate to these questions both in the intact follicle *in vivo*, as well as in primary GC cultures and in established GC lines.

The intact follicle

The ovarian follicle, the basic functional unit of the ovary, consists of outer layers of theca cells which encircle inner layers of GCs. The outer boundary of the GC layers is separated from blood vessels and theca cells by a basement membrane lining the follicle. GCs from the inner layer surround the innermost oocyte-cumulus cell complex (Fig. 1). The maturation of ovarian follicles and their transformation into the highly steroidogenic corpora lutea is regulated by pituitary gonadotropin secretion in a cyclic manner^{1–3}.

In response to gonadotropin secretion various follicular compartments interact in a highly integrated manner to secrete the sex steroids

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The Poly(A)-Poly(A)-Binding Protein Complex Is a Major Determinant of mRNA Stability In Vitro

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Using an in vitro mRNA decay system, we investigated how poly(A) and its associated poly(A)-binding protein (PABP) affect mRNA stability. Cell extracts used in the decay reactions were depleted of functional PABP either by adding excess poly(A) competitor or by passing the extracts over a poly(A)-Sepharose column. Polyadenylated mRNAs for β -globin, chloramphenicol acetyltransferase, and simian virus 40 virion proteins were degraded 3 to 10 times faster in reactions lacking PABP than in those containing excess PABP. The addition of purified *Saccharomyces cerevisiae* or human cytoplasmic PABP to PABP-depleted reactions stabilized the polyadenylated mRNAs. In contrast, the decay rates of nonpolyadenylated mRNAs were unaffected by PABP, indicating that both the poly(A) and its binding protein were required for maintaining mRNA stability. A nonspecific single-stranded binding protein from *Escherichia coli* did not restore stability to polyadenylated mRNA, and the stabilizing effect of PABP was inhibited by anti-PABP antibody. The poly(A) tract was the first mRNA segment to be degraded in PABP-depleted reactions, confirming that the poly(A)-PABP complex was protecting the 3' region from nucleolytic attack. These results indicate that an important function of poly(A), in conjunction with its binding protein, is to protect polyadenylated mRNAs from indiscriminate destruction by cellular nucleases. A model is proposed to explain how the stability of an mRNA could be affected by the stability of its poly(A)-PABP complex.

Most newly synthesized mRNA molecules contain a poly(A) tract when they enter the cytoplasm of a nucleated cell. Therefore, poly(A) is presumed to play a significant role in mRNA synthesis, translation, and/or metabolism. However, the exact functions of poly(A) and its associated poly(A)-binding protein (PABP) are incompletely understood. Poly(A) is potentially involved in any of four stages of mRNA metabolism: splicing, nucleus-to-cytoplasm transport, translation, and stability (reviewed in references 47 and 50). Although polyadenylation and splicing are linked, they can occur independently (72), and polyadenylation is not an absolute prerequisite for transport, because some nonadenylated RNAs are transported efficiently (3, 26, 73). Some viral mRNAs are synthesized exclusively in the cytoplasm and are polyadenylated, indicating an extranuclear role for poly(A) (1, 5, 19). There is evidence for a link between poly(A) and translation, especially in lower organisms (16-18, 21, 47, 62), and some experiments suggest that poly(A) affects mRNA half-lives (see below).

The role of PABP in mRNA metabolism is also unclear, although its highly specific binding properties imply that its function is linked with at least one function of poly(A) (9, 35). The molecular weight of cytoplasmic PABP varies from 72,000 in mammals (9, 23) to 68,000 in *Saccharomyces cerevisiae* (2, 58, 59) to 31,000 in slime molds (36, 37). Yeast and human PABPs share a high degree of homology within the N-terminal region, which is responsible for the poly(A)-binding function. They diverge in the C-terminal third of the protein (23). PABP interacts with poly(A), forming an ordered, nucleosomelike complex with a periodicity of 25 to 27 nucleotides, and its affinity for poly(A) is at least 100-fold greater than that for most other polynucleotides (6, 7).

Despite its high affinity for poly(A), it can exchange or migrate from one poly(A) molecule to another (59), a property which might be important in terms of its proposed function as a determinant of mRNA stability (see Discussion). Deletion of *PAB* (the PABP gene) from the yeast *S. cerevisiae* is lethal, indicating that PABP is an essential protein (59).

Some experiments with intact cells provide compelling, but indirect, evidence that poly(A) metabolism is linked with mRNA stability (reviewed in references 10, 51, 53, and 61). For example, the poly(A) tracts of some mRNAs are shortened and ultimately removed in a time-dependent manner, implying that poly(A) removal precedes degradation of the mRNA body (13, 25, 39, 52, 70). This observation, in turn, has strengthened the notion that poly(A) protects some mRNAs from rapid, indiscriminate degradation. However, direct evidence supporting a poly(A) protection function has been elusive, for several reasons. First, inhibitors that interfere with polyadenylation can affect other ATP-dependent processes and, therefore, lack complete specificity. Second, it has been difficult to exploit genetic approaches for generating full-length, deadenylated mRNAs in cells. Mutations in genomic polyadenylation signals can interfere with polyadenylation when the mutated genes are transfected into cells, but the resulting transcripts usually bear little resemblance to the wild-type mRNA. They are frequently elongated past the normal 3' terminus and have heterogeneous 3' ends (69).

In view of these and other limitations with experiments using intact cells, we have exploited an in vitro mRNA decay system to investigate how poly(A) and PABP affect mRNA turnover rates. An in vitro system provides some significant advantages over whole cells, because the decay of synthetic mRNAs, polyadenylated or not, can be assayed rapidly and

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quantitatively without the use of complicated pulse-chase labeling techniques. Furthermore, PABP levels can be varied systematically, permitting the functions of poly(A) and PABP to be analyzed independently. The mRNA decay system that we have described includes either polysomes or a soluble, high-salt extract of polysomes (54, 55). The mRNA-degrading enzymes are associated with the polysomes and are solubilized by incubation of the polysomes in a buffer containing 0.3 to 0.5 M KCl (55). The polysomes are then pelleted, and the supernatant or ribosomal salt wash (RSW) fraction is harvested. Both polysomes and the RSW fraction contain PABP.

Several observations indicate that this system is a valid model for studying mRNA turnover and poly(A) function. (i) The rank order of mRNA decay rates in cells is reflected in vitro. For example, *c-myc* and histone mRNAs are degraded rapidly in cells and in vitro, whereas β - and γ -globin mRNAs are stable in both (54, 56). (ii) Histone mRNAs are degraded in a 3'-to-5' direction by one or more exonucleases, both in cells and in vitro, and the decay intermediates generated in vitro are indistinguishable from those observed in intact cells (56). Therefore, mRNA decay pathways are reproduced faithfully in vitro. (iii) The effects of poly(A) on mRNA turnover in vitro are similar to those in whole cells. For example, polyadenylated histone mRNA is at least 10-fold more stable than its deadenylated counterpart in vitro and in cells (48; reviewed in reference 38). (iv) Recent in vitro data support the observation that poly(A) removal can precede degradation of the mRNA body. The first step in the in vitro decay of *c-myc* mRNA is poly(A) removal (11). The second step is degradation of the mRNA body, and this step does not begin until most or all of the poly(A) tract is removed. In contrast, both the poly(A) and the body of a stable mRNA, γ -globin, remain intact over long incubation times. These observations suggest that poly(A) can protect mRNA from nucleolytic attack. Once the poly(A) is removed, the mRNA body can be degraded.

These results also raised three questions regarding the relationship between poly(A) and mRNA stability. (i) Is the stability of an mRNA molecule proportional to the stability of its poly(A) tract? As discussed above, an unstable mRNA had an unstable poly(A) tract in vitro, while a stable mRNA had a stable poly(A) tract (11). (ii) Does poly(A) protect the 3' regions of mRNAs from nuclease attack, and does it thereby contribute significantly in determining the half-lives of individual mRNAs? (iii) If so, what factors account for differential poly(A) shortening among different mRNAs? How can the poly(A) tract of an unstable mRNA be degraded faster than that of a stable mRNA when all poly(A) tracts have the same primary structure, disregarding size differences? The experiments presented here were designed primarily to investigate the second question.

We find that polyadenylated mRNAs which are normally stable in vitro become unstable when PABP is removed from the reaction mixture. Adding back cytoplasmic PABP restores stability, and PABP has no effect on mRNAs lacking poly(A). These results indicate that the poly(A)-PABP complex, not poly(A) alone, protects at least some mRNAs from indiscriminate destruction. Therefore, the most important feature of mRNA turnover, namely, that different mRNAs are degraded at different rates, is dependent on both components.

MATERIALS AND METHODS

The culturing of K562 human erythroleukemia cells and the preparation of RSW and 32 P-labeled human H4 histone

and β -globin mRNAs have been described previously (54, 55). The polyadenylated simian virus 40 (SV40) and chloramphenicol acetyltransferase (CAT) mRNAs were synthesized with SP6 RNA polymerase to transcribe cDNA clones kindly provided by Peter Good, Janet Mertz, and Judy Callis. The in vitro mRNA decay reaction conditions and methods for mRNA analysis and densitometric scanning were as previously described (55). RSW (0.3 M KCl) was used for all of the reactions described here. Each 25- μ l reaction contained 4 μ g of RSW protein, corresponding to approximately 7×10^6 cells. The H mapping or oligonucleotide-directed RNase H procedure was performed as described by Brewer and Ross (11). Deadenylated β -globin mRNA was prepared by incubating the 32 P-labeled substrate with oligo(dT) plus RNase H (11).

S. cerevisiae PABP (p68) was prepared and kindly provided by Alan Sachs and Roger Kornberg. The protein was purified by A. Sachs and R. Kornberg from an *Escherichia coli* strain expressing the yeast p68 PAB gene (58, 59). Where indicated, yeast or human PABP was mixed on ice with the in vitro mRNA decay reaction components. The reaction mixtures were warmed to 37°C and incubated for various times. Both the preimmune immunoglobulin and the yeast anti-PABP antibody (anti-p68) were prepared and kindly provided by David Munroe, Richard Manrow, and Allan Jacobson. Each immunoglobulin was purified by D. Munroe, R. Manrow, and A. Jacobson by ammonium sulfate precipitation and chromatography on DEAE-cellulose and carboxymethyl cellulose. Purified immunoglobulin was preincubated with PABP on ice for 30 min. The mixture was added to chilled mRNA decay reaction mixtures, which were then mixed and brought to 37°C. For protein blotting, proteins were electrophoresed in a 10% sodium dodecyl sulfate-polyacrylamide minigel (Idea Scientific Co.) and electroblotted to nitrocellulose. The blot was incubated for 30 min in a solution of 3% (wt/vol) bovine serum albumin, washed, and incubated at 4°C for 16 h with 20 μ g of purified immunoglobulin. After being washed, the blot was incubated for 3 h at 25°C with a 1:250 dilution of goat anti-rabbit immunoglobulin G-peroxidase conjugate (Sigma Chemical Co.). It was then developed by incubation for 5 min at room temperature with a 1:1 mixture of hydrogen peroxide and 4-chloro-1-naphthol solution (purchased from Kirkegaard and Perry) and photographed after being washed with water.

PABP-depleted RSW was prepared at 4°C as follows. Dry poly(A)-Sepharose (100 mg; Pharmacia, Inc.) was suspended in 2 ml of buffer A (500 mM KCl, 10 mM EDTA, 10 mM Tris chloride [pH 7.5]). The material was placed in a disposable plastic column (Bio-Rad Laboratories) and washed at room temperature, first with 10 ml of buffer B (10 mM KCl, 10 mM EDTA, 10 mM Tris chloride [pH 7.5]), then with 5 ml of phosphate-buffered formamide (20 mM phosphate [pH 6.8]), and then with 5 ml of buffer B. The column was placed in a cold room. A sample of the RSW from approximately 6×10^8 cells (325 μ g of protein in 0.25 ml) was dialyzed at 4°C against 500 ml of buffer B, with one buffer change. The dialyzed RSW was applied to the column, which was washed with 5 ml of buffer B and then with 5 ml of buffer A. The original flowthrough plus the two 5-ml washes were pooled and concentrated to approximately 0.25 ml by centrifugation in a Centricon tube with a 10,000-molecular-weight cutoff (Amicon Corp.). As determined by the 32 P-poly(A)-binding assay (60), approximately 90% of the PABP was removed from the crude RSW by this procedure. Minor differences in several high-molecular-weight (>200,000) stained proteins were observed when crude RSW was compared with PABP-

depleted RSW by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). However, as determined by the decay of the ^{32}P -mRNAs, 80 to 100% of the mRNA-degrading activity was recovered in the PABP-depleted RSW (see Results). In some experiments, the poly(A)-Sephacose column was further washed with 2 M guanidine hydrochloride-30 mM Tris (pH 7.5) in an effort to elute the bound PABP. However, active PABP was not recovered after renaturation of the eluates.

Partially purified human PABP was prepared by techniques described for yeast PABP (60). All buffers included the following protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 0.4 μg of leupeptin per ml, and 1.4 μg of pepstatin per ml. The starting material was polysomes from exponentially growing K562 cells. Polysomes from 3×10^9 cells (18 mg of protein) were mixed with 500 μg of poly(A) and 5 mg of poly(C) (Pharmacia) in adsorption buffer (100 mM NaCl, 30 mM Tris hydrochloride [pH 8.0]). The material was applied at 25°C to 1 g of oligo(dT)-cellulose (Collaborative Research, Inc.) in a water-jacketed column. The column was washed with 10 column volumes of high-salt buffer (330 mM NaCl, 30 mM Tris chloride [pH 8.0]), and poly(A) and bound material were eluted by washing the column with 2 column volumes of low-salt buffer (5 mM EDTA, 5 mM Tris hydrochloride [pH 8.0]) at 45°C. The low-salt eluate was chilled on ice and applied to a 2-ml cibacron blue column (Pierce Chemical Co.) in a cold room. The column was washed with 10 column volumes of PABP-binding buffer (PBB; 100 mM NaCl, 5 mM EDTA, 15 mM β -mercaptoethanol, 30 mM Tris chloride [pH 8.0]), then with 3 volumes of PBB containing 4 M NaCl rather than 100 mM NaCl, then with 3 volumes of PBB, and then with 10 volumes of PBB containing 0.5 M guanidine hydrochloride. PABP was eluted in 5 ml of buffer G (100 mM NaCl, 5 mM EDTA, 15 mM β -mercaptoethanol, 2 M guanidine hydrochloride, 30 mM Tris chloride [pH 8.0]). PABP activity was restored by diluting the eluate with PBB. These steps resulted in a purification of approximately 60-fold and a recovery of 350 μg of protein. PABP was assayed by the ^{32}P -poly(A)-binding assay (60).

E. coli single-stranded DNA-binding protein (SSB) was purchased from U.S. Biochemical Corp. The globin antisense oligodeoxynucleotide was prepared at the University of Wisconsin Biotechnology Center. Ribohomopolymers were purchased from Pharmacia, and their lengths were verified by denaturing polyacrylamide gel electrophoresis and methylene blue staining.

RESULTS

Depletion of PABP by exogenous competitor poly(A) destabilizes polyadenylated mRNA. The following observations with an *in vitro* mRNA decay system suggested that the poly(A)-PABP complex might protect some mRNAs from rapid destruction. (i) Deadenylated, ^{32}P -labeled mRNAs were at least 10-fold less stable than polyadenylated mRNAs (48; see also references 8, 18, 28, 29, and 33). (ii) The reactions contained sufficient excess PABP to bind all of the poly(A) on the ^{32}P -labeled mRNA substrates (P. Bernstein, unpublished data). To investigate directly whether the poly(A)-PABP complex affected mRNA stability *in vitro*, we asked if polyadenylated mRNAs that were normally stable became unstable in reactions depleted of PABP. Two methods were exploited to extract PABP from the RSW, which was used as the source of mRNA-degrading activity for these experiments: adding excess competitor poly(A) to the

reactions or passing the RSW through a poly(A)-Sephacose column. Competitor poly(A) sequesters the PABP so that little or none is available to interact with the ^{32}P -labeled mRNAs. Poly(A)-Sephacose binds PABP with high selectivity and thereby extracts it from the RSW.

Increasing amounts of all four ribohomopolymers (each 1,000 to 2,000 nucleotides in length) were added separately to *in vitro* mRNA decay reactions containing RSW plus polyadenylated β -globin and nonpolyadenylated H4 histone ^{32}P -mRNAs. Reactions were incubated for 20 min at 37°C, and RNA was purified and electrophoresed. As described previously (54), β -globin mRNA was stable and histone mRNA was unstable in reactions without ribohomopolymers (Fig. 1A, lane 3). As little as 6 to 30 ng of competitor poly(A) induced destabilization of the β -globin mRNA but did not affect histone mRNA decay (Fig. 1A, lanes 4 to 6). None of the other ribohomopolymers had a comparable effect on the polyadenylated mRNA (Fig. 1A, lanes 7 to 15). Poly(G) at 150 ng per reaction destabilized it to some extent in this experiment, but the effect was too small to observe consistently (Fig. 1B). Kinetic analysis confirmed that poly(A) affected the polyadenylated substrate specifically (Fig. 1B, compare lanes 2 to 7 and 8 to 13). As estimated by scanning densitometry, it was degraded seven times faster with poly(A) than with poly(G) or no homopolymer.

Two classes of globin mRNA decay intermediates were observed in reactions with competitor poly(A). One was heterogeneous and migrated between the full-length mRNA and the deadenylated mRNA (Fig. 1B, lanes 3 to 7). We show later that this class corresponded to mRNA molecules whose poly(A) tracts were being shortened (see Fig. 9). The second intermediate, observed most clearly in Fig. 1B, lanes 6 and 7, migrated as a more discrete band at the same position as deadenylated mRNA [A(-) β]. Still smaller products were either not observed or were scarce, suggesting that the deadenylated product resulted from a holdup point in the decay process. These intermediates suggest a stepwise decay pathway in which the poly(A) is first shortened and then removed and, after a brief holdup, the deadenylated mRNA is then completely destroyed. We do not know the reason for the holdup.

Yeast cytoplasmic PABP overcomes the destabilizing effect of competitor poly(A). Since poly(C), poly(G), and poly(U) had little or no effect on the decay of the polyadenylated mRNA, it seemed likely that competitor poly(A) was sequestering the PABP in the RSW. As a result, insufficient PABP was available to bind to the ^{32}P -mRNA, whose protein-free poly(A) tract was then destroyed, permitting the mRNA body to be degraded soon thereafter. Therefore, we asked if exogenous PABP could restabilize the mRNA in the presence of competitor poly(A). Cytoplasmic PABP was purified from an *E. coli* strain expressing the yeast PABP gene (*PAB*) and was kindly provided by Alan Sachs and Roger Kornberg (58, 59). As described above, the polyadenylated mRNA was stable without competitor poly(A) but was unstable with it (Fig. 2A, compare lanes 2 and 3). Yeast cytoplasmic PABP (p68) stabilized the mRNA, even in the presence of competitor poly(A) (Fig. 2A, lanes 4 to 7). Maximal stabilization was reached with approximately 2 μg of PABP per reaction but was evident with 0.5 μg . Since each yeast PABP molecule binds 25 adenylate residues (59), each mole of homopolymer (average length, 1,000 to 2,000 residues) should bind at least 40 mol of PABP. Therefore, PABP at 2 μg per reaction represented a maximum two- to threefold molar excess over the theoretical number of PABP-binding sites available in 150 ng of competitor poly(A). mRNA

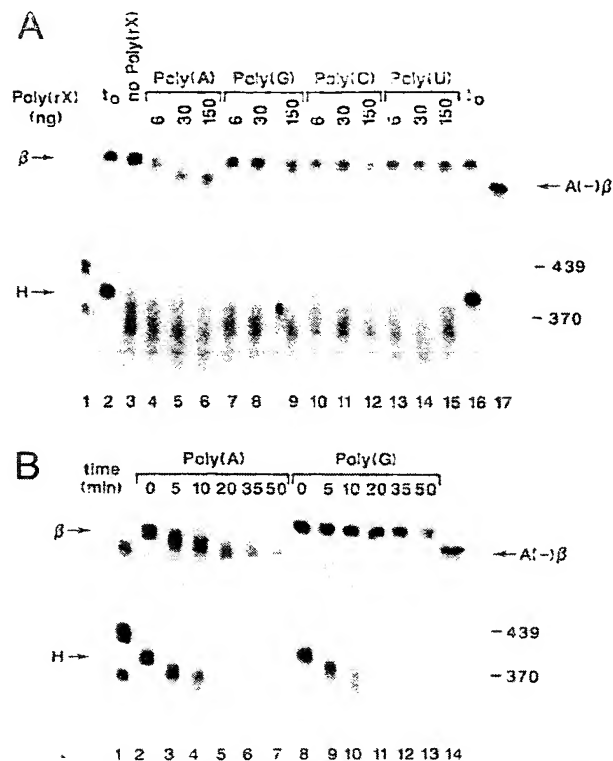


FIG. 1. Effect of competitor poly(A) on the stability of β -globin and histone ^{32}P -mRNAs. In vitro mRNA decay reactions (25- μl volumes) containing 0.3 M KCl RSW, ^{32}P -labeled human H4 histone (nonpolyadenylated), and ^{32}P -labeled β -globin (polyadenylated) mRNAs and the indicated amounts of each ribohomopolymer were incubated for 0 or 20 min at 37°C. RNAs were extracted and electrophoresed in 4% polyacrylamide-7 M urea gels. (A) Comparison of the effects of four ribohomopolymers. Lane 1: Marker pBR322 DNA cleaved with *Hae*II and labeled with polynucleotide kinase; the sizes in nucleotides of some of the fragments are noted on the right. Lanes 2 and 16: Unincubated reactions (t_0); the locations of undegraded β -globin and histone mRNAs are indicated by β and H, respectively. Lane 3: Reaction incubated for 20 min at 37°C with no ribohomopolymer. Lanes 4 to 15: Reactions incubated for 20 min at 37°C with various amounts (nanograms per reaction) of ribohomopolymer. Lane 17: Deadenylated β -globin mRNA marker [A(-) β], prepared by incubating polyadenylated β -globin mRNA with oligo(dT) and RNase H (see Materials and Methods). (B) Time course of mRNA degradation in reactions with poly(A) or poly(G). mRNA decay reactions containing 150 ng of poly(A) or poly(G) were incubated for the indicated times, and ^{32}P -RNAs were electrophoresed as in panel A. Lane 1: DNA marker, as in panel A. Lanes 2 to 13: Reactions containing poly(A) or poly(G) incubated for the indicated times (minutes). Lane 14: Deadenylated β -globin mRNA marker, as in panel A. β and H are as in panel A.

stability was determined by the ratio of poly(A) to PABP, because fivefold more PABP was required to stabilize the mRNA when fivefold more competitor poly(A) was added (data not shown). Kinetic analysis confirmed that exogenous PABP restored the stability of the polyadenylated ^{32}P -mRNA (Fig. 2B), which was degraded six times faster without PABP (lanes 6 to 10) than with it (lanes 11 to 15).

Depleting the RSW of the endogenous PABP destabilizes polyadenylated mRNA. The RSW was chromatographed

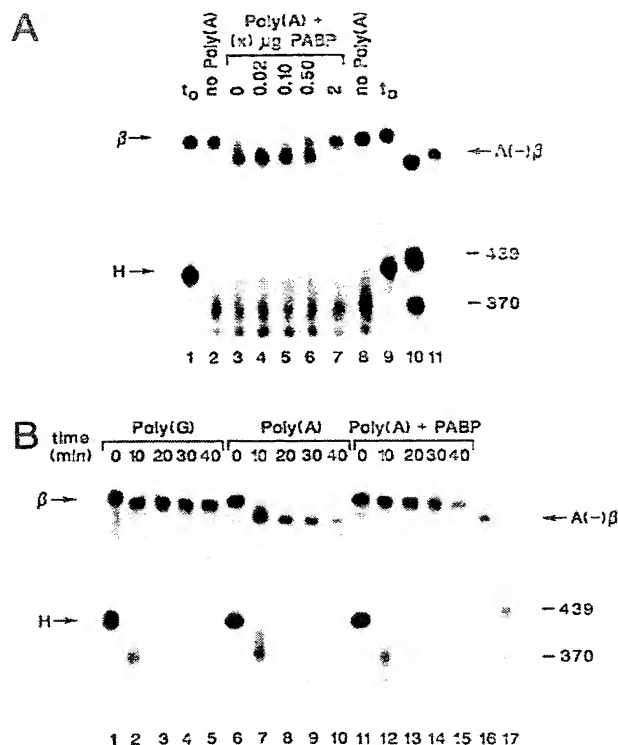


FIG. 2. Effect of purified yeast cytoplasmic PABP on the stability of β -globin mRNA. mRNA decay reactions contained polyadenylated β -globin and nonpolyadenylated H4 histone ^{32}P -mRNA substrates. In vitro mRNA decay reactions were incubated for the indicated times with or without 150 ng of competitor poly(A) and, where noted, with increasing amounts of purified, *E. coli*-derived yeast cytoplasmic PABP (59). RNAs were extracted and analyzed by electrophoresis in 4% polyacrylamide-7 M urea gels. (A) Effect of increasing amounts of PABP. Lanes 1 and 9: Unincubated reactions (t_0); undegraded β -globin and H4 histone mRNAs are indicated by β and H, respectively. Lanes 2 and 8: Reactions incubated for 20 min without poly(A) and without PABP. Lanes 3 to 7: Reactions containing 150 ng of competitor poly(A) and the indicated amounts (micrograms) of PABP. Lane 10: Marker pBR322 DNA cleaved with *Hae*II; fragment sizes in nucleotides are noted on the right. Lane 11: Deadenylated β -globin mRNA marker [A(-) β], as in Fig. 1. (B) Time course. Lanes 1 to 5: Reactions containing 150 ng of poly(G) and 2 μg of bovine serum albumin. Lanes 6 to 10: Reactions containing 150 ng of poly(A) and 2 μg of bovine serum albumin. Lanes 11 to 15: Reactions containing 150 ng of poly(A) and 2 μg of yeast PABP. Incubation times (minutes) for lanes 1 to 15 are noted at the top. Lane 16: Deadenylated β -globin mRNA marker, as in panel A. Lane 17: DNA marker, as in panel A. β and H are as in panel A.

over a poly(A)-Sepharose column to deplete it of PABP. As determined by a ^{32}P -poly(A)-binding assay (60), the chromatographed RSW contained approximately 10-fold less PABP than did the native RSW. Polyadenylated β -globin and nonpolyadenylated histone mRNAs were both unstable in reactions containing PABP-depleted RSW (Fig. 3A, lane 4). Therefore, PABP depletion destabilized the polyadenylated mRNA but did not inactivate messenger RNase activity. When purified yeast cytoplasmic PABP was added to the

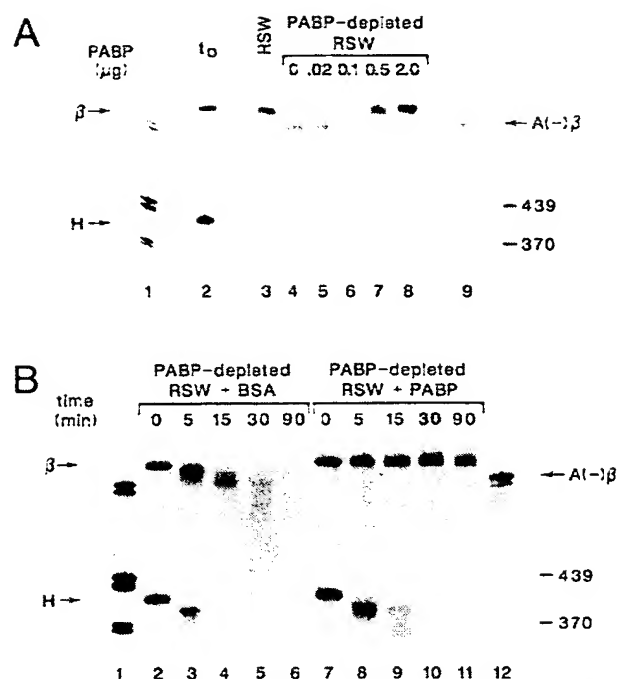


FIG. 3. Stabilization of β -globin mRNA by the addition of yeast cytoplasmic PABP to PABP-depleted RSW. The RSW was chromatographed over poly(A)-Sepharose to extract PABP (see Materials and Methods). PABP-depleted RSW was incubated under standard reaction conditions with β -globin and H4 histone 32 P-mRNAs and with or without purified yeast cytoplasmic PABP. RNAs were extracted and electrophoresed in 4% polyacrylamide-7 M urea gels. (A) Effect of PABP concentration on mRNA stability. Reactions were incubated for 20 min at 37°C, unless otherwise noted. Lane 1: Marker pBR322 DNA cleaved with *Hae*III; fragments sizes in nucleotides are noted on the right. Lane 2: Unincubated reaction (t₀); undegraded β -globin and H4 histone mRNAs are indicated by β and H, respectively. Lane 3: Control reaction with PABP-containing RSW that had not been chromatographed over poly(A)-Sepharose. Lane 4: Reaction with PABP-depleted RSW and 2 μ g of bovine serum albumin and without PABP. Lanes 5 to 8: Reactions with PABP-depleted RSW plus increasing amounts (micrograms per reaction) of purified yeast PABP; all reactions contained a total of 2 μ g of added protein, consisting of PABP and bovine serum albumin, when necessary. Lane 9: Deadenylated β -globin mRNA [A(-) β], as in Fig. 1. (B) Time course of effect of PABP on mRNA incubated with PABP-depleted RSW. Lane 1: DNA marker, as in panel A. Lanes 2 to 6: PABP-depleted RSW plus 2 μ g of bovine serum albumin (BSA). Lanes 7 to 11: PABP-depleted RSW plus 2 μ g of yeast PABP. Incubation times (minutes at 37°C) for lanes 2 to 11 are noted at the top. Lane 12: Deadenylated β -globin mRNA marker, as in panel A. β and H are as in panel A.

depleted RSW, the polyadenylated mRNA became stable (Fig. 3A, lanes 5 to 8), confirming that the mRNA had become unstable because the PABP had been removed. Kinetic analysis indicated that PABP stabilized the polyadenylated substrate approximately 20-fold (Fig. 3B). It is significant that histone mRNA decay rates were similar with untreated RSW and with PABP-depleted RSW supplemented with yeast PABP (Fig. 3B). This result indicates that the depletion step did not significantly modify the mRNA-degrading enzymes or other cofactors besides PABP.

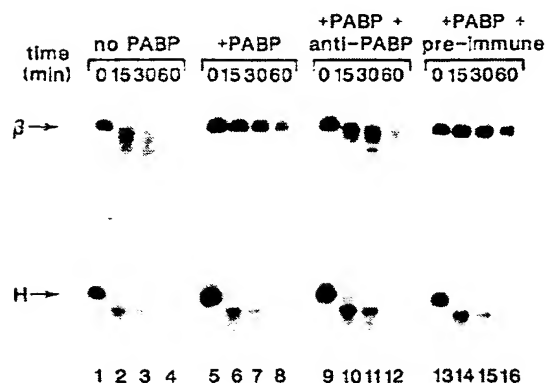


FIG. 4. Blockage by anti-p68 of stabilization of β -globin mRNA by yeast cytoplasmic PABP. All reaction mixtures contained PABP-depleted RSW. Yeast cytoplasmic PABP or bovine serum albumin was preincubated with purified anti-p68 or preimmune immunoglobulin on ice for 30 min before the other mRNA decay reaction components were added. Incubation times (minutes) at 37°C are noted at the top. RNAs were extracted and electrophoresed in 4% polyacrylamide-7 M urea gels. β and H indicate the positions of undegraded β -globin and H4 histone 32 P-mRNAs, respectively. Lanes 1 to 4: 10.5 μ g of bovine serum albumin. Lanes 5 to 8: 10 μ g of bovine serum albumin plus 500 ng of yeast PABP. Lanes 9 to 12: 10 μ g of anti-p68 plus 500 ng of PABP. Lanes 13 to 16: 10 μ g of preimmune immunoglobulin plus 500 ng of PABP.

Antibody to yeast PABP blocks its ability to stabilize polyadenylated mRNA. To confirm that the exogenous PABP was actually responsible for restabilizing the mRNA, we attempted to block formation of the poly(A)-PABP complex by preincubating yeast cytoplasmic PABP (p68) with purified anti-p68. Highly specific anti-p68 (see Fig. 5) was raised in rabbits with purified yeast PABP from Alan Sachs and Roger Kornberg (58, 59) as the immunogen. The antibody was prepared, purified, and kindly provided by David Munroe, Richard Manrow, and Allan Jacobson. PABP was preincubated with bovine serum albumin, preimmune immunoglobulin, or anti-p68. The mixtures were added to reactions containing PABP-depleted RSW and 32 P-mRNAs, the reactions were incubated at 37°C, and the RNAs were analyzed by gel electrophoresis. The polyadenylated substrate was unstable without PABP but was stabilized by it (Fig. 4, compare lanes 1 to 4 and 5 to 8). Anti-p68, but not preimmune immunoglobulin, interfered with the stabilization effect (Fig. 4, lanes 9 to 16). Antibody neutralization was not complete (Fig. 4, compare lanes 9 to 12 and 1 to 4), probably because the antibody was unable to react with all of the PABP in the reaction. Both anti-p68 and preimmune immunoglobulin slowed histone mRNA degradation slightly, but they did so to approximately the same extent. Neither had any detectable nuclease activity in reactions lacking RSW (data not shown).

To assess the specificity of the antibody and its capacity to cross-react with RSW proteins, we electrophoresed and blotted control RSW or RSW supplemented with yeast PABP and reacted the blot with anti-p68 or preimmune immunoglobulin. A prominent band was observed in the lanes containing yeast PABP but not in the lanes containing RSW alone (Fig. 5). Therefore, anti-p68 reacted specifically with yeast cytoplasmic PABP and not with components in the RSW.

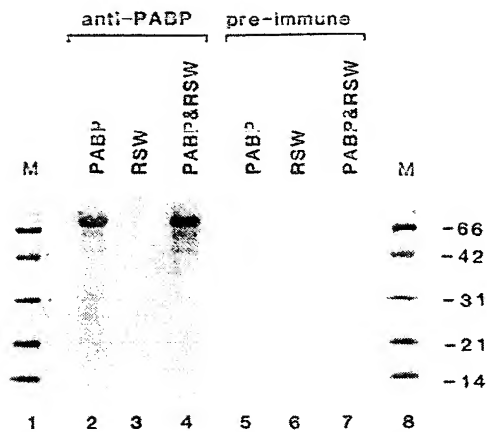


FIG. 5. Western blotting (immunoblotting) analysis of RSW and RSW supplemented with yeast PABP. Proteins were electrophoresed in a 10% sodium dodecyl sulfate-polyacrylamide gel and were blotted as described in Materials and Methods. The blots were incubated with either purified anti-p68 or purified preimmune immunoglobulin, as noted at the top. Antigen-antibody complexes were visualized by peroxidase staining. Lanes 1 and 8: Marker proteins; molecular weights (in thousands) are indicated on the right. Lanes 2 and 5: 2 μ g of yeast cytoplasmic PABP (p68). Lanes 3 and 6: 15 μ g of PABP-depleted RSW plus 2 μ g of bovine serum albumin. Lanes 4 and 7: 15 μ g of PABP-depleted RSW plus 2 μ g of yeast cytoplasmic PABP.

Partially purified human PABP protects polyadenylated mRNA from rapid degradation. Human PABP was partially purified by the method of Sachs and Kornberg (60). The steps (Fig. 6A legend and Materials and Methods) included chromatography on oligo(dT)-cellulose and cibacron blue (Fig. 6A), but overloading of gels revealed several additional minor bands. The preparation was estimated to be 90% pure. β -Globin mRNA was degraded at least four times more slowly in the presence than in the absence of human PABP (Fig. 6B). In this experiment and others as well, the extent of protection was similar with the human and yeast PABPs.

Poly(A) and PABP affect mRNA stability via specific interactions. To confirm that PABP stabilized the polyadenylated mRNA by binding to the 3'-terminal poly(A), we asked if PABP would stabilize deadenylated globin mRNA in reactions with PABP-depleted RSW. The polyadenylated mRNA was stabilized by PABP (Fig. 7, compare lanes 2 to 4 and 5 to 7), but the deadenylated substrate, which was identical to the polyadenylated mRNA except for the missing poly(A) tract, was degraded rapidly, and its decay was unaffected by PABP (lanes 8 to 13). Furthermore, the deadenylated β -globin mRNA had the same half-life in reactions containing or lacking exogenous poly(A) (data not shown). Thus, the normally large difference in the turnover rates of histone and globin mRNAs was not maintained when either poly(A) or PABP was missing. Moreover, the effect of PABP on mRNA decay was dependent on the presence of a poly(A) tract.

The apparent requirement for both poly(A) and PABP to maintain stability implied that unprotected mRNA was first attacked at its 3' end, that is, at its poly(A) tract. To determine the pathway of mRNA decay under PABP-deficient conditions, we exploited an oligonucleotide-directed

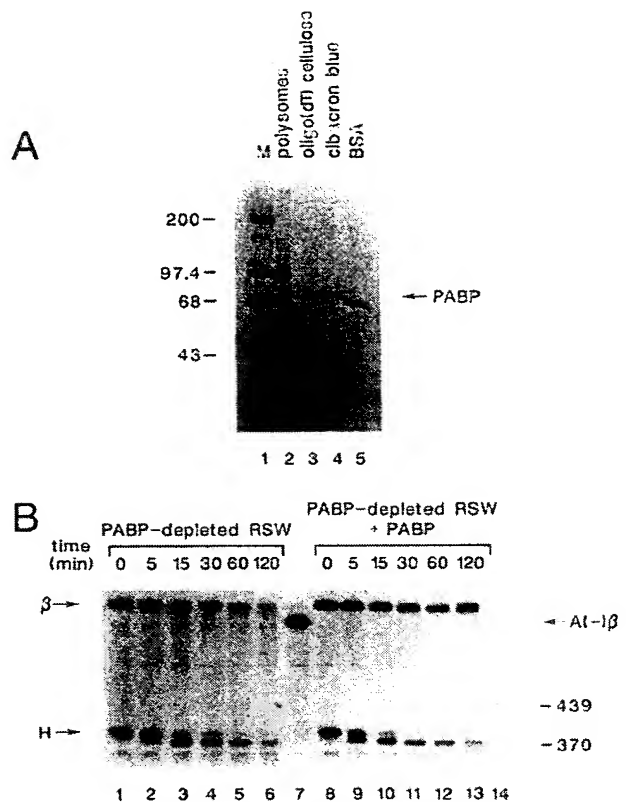


FIG. 6. Stabilization of polyadenylated mRNA by partially purified human PABP in PABP-depleted RSW. (A) Purification of PABP. Human PABP was partially purified from K562 cell polysomes (see Materials and Methods). One percent of the material from each purification step was electrophoresed in an 8% sodium dodecyl sulfate-polyacrylamide gel, which was stained with Coomassie blue. The arrow on the right indicates the PABP band. Lane 1: Molecular weight markers; sizes (in thousands) are indicated on the left. Lane 2: Starting material (proteins from K562 cell polysomes). Lane 3: Material eluted in low-salt buffer from oligo(dT)-cellulose. Lane 4: Material eluted from cibacron blue. Lane 5: Bovine serum albumin (BSA) marker. (B) mRNA stabilization by human PABP. In vitro mRNA decay reactions contained β -globin and H4 histone 32 P-mRNAs plus PABP-depleted RSW and were incubated for the indicated times (minutes) at 37°C. RNAs were electrophoresed in 3% polyacrylamide-7 M urea gels. The locations of polyadenylated (β) and deadenylated [$A(-)\beta$] globin mRNAs and H4 histone (H) mRNA are noted. Lanes 1 to 6: 2.5 μ g of bovine serum albumin added to each reaction. Lane 7: Deadenylated β -globin mRNA, as in Fig. 1. Lane 8 to 13: 2.5 μ g of partially purified human PABP (cibacron blue step) added to each reaction. Lane 14: pBR322 DNA *Hae*III marker; fragment sizes in nucleotides are noted on the right.

RNAse H cleavage method (H mapping) that had been useful for analyzing the early steps in *c-myc* mRNA decay (11) (Fig. 8). Polyadenylated β -globin 32 P-mRNA was incubated in reactions containing nondepleted RSW plus 150 ng of either poly(A) or poly(G). Total RNA was extracted and annealed with a single-stranded oligodeoxynucleotide (25-mer) complementary to a region located between 106 and 130 nucleotides 5' of the globin mRNA poly(A) addition site (Fig. 8).

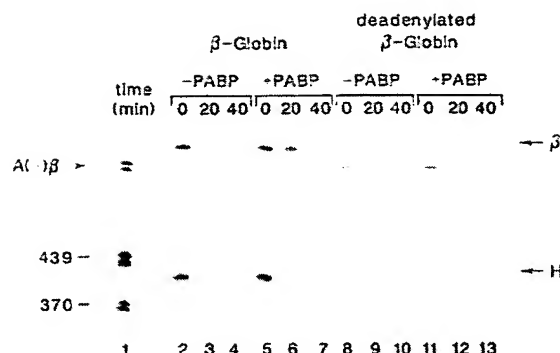


FIG. 7. Effect of yeast cytoplasmic PABP on the decay of polyadenylated versus nonpolyadenylated β -globin mRNA. In vitro mRNA decay reactions contained PABP-depleted RSW, H4 histone (H) 32 P-mRNA, and either polyadenylated (β) or nonpolyadenylated [$A(-)\beta$] β -globin 32 P-mRNA. Where indicated, 2 μ g of yeast PABP was added. Reactions were incubated for the indicated times (minutes), and RNAs were extracted and electrophoresed in 4% polyacrylamide-7 M urea gels. Lane 1: Marker pBR322 DNA cleaved with *Hae*III; fragment sizes in nucleotides are noted on the left. Lanes 2 to 7: Polyadenylated β -globin mRNA (β). Lanes 8 to 13: Deadenylated β -globin mRNA [$A(-)\beta$].

The DNA-RNA hybrids were treated with RNase H, which generated two fragments, corresponding to 5' and 3' mRNA segments. If degradation were occurring 3' to 5', beginning in the poly(A) tract, the smaller 3' fragment should have been degraded sooner than the larger 5' one. Undegraded mRNA generated the two expected fragments (Fig. 9A, lanes 1 and 13). The size of the smaller fragment, labeled 3'- β , corresponded to the 3'-terminal 106 to 120 nucleotides of the mRNA plus the 80-nucleotide poly(A) tract. The difference between the polyadenylated and deadenylated 3'- β fragments is shown in Fig. 9A, lanes 13 and 14. The 3' fragment was less stable in reactions with poly(A) than in those with poly(G) (Fig. 9A). More importantly, the 3' fragment was destroyed faster than the 5' fragment (Fig. 9B). Therefore, poly(A) was the first part of the mRNA to be degraded in PABP-depleted reactions, confirming that PABP

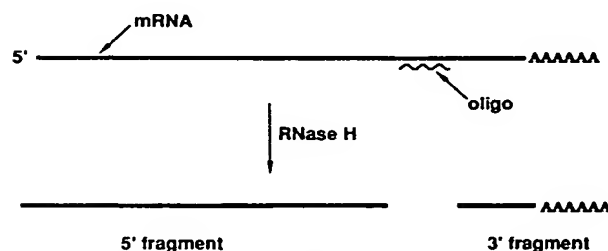


FIG. 8. RNase H mapping method for analyzing poly(A) shortening and for determining the directionality of mRNA decay. Details of the method have been described previously (11). Briefly, a synthetic antisense oligodeoxyribonucleotide is annealed to the 32 P-mRNA, and the hybrid is treated with RNase H, generating 5' and 3' mRNA fragments. The fragments are then electrophoresed. Since site-specific RNase H cleavage defines the 5'-terminal region of the 3' fragment, small changes in the size of that fragment that occur during the in vitro incubation must result from poly(A) shortening.

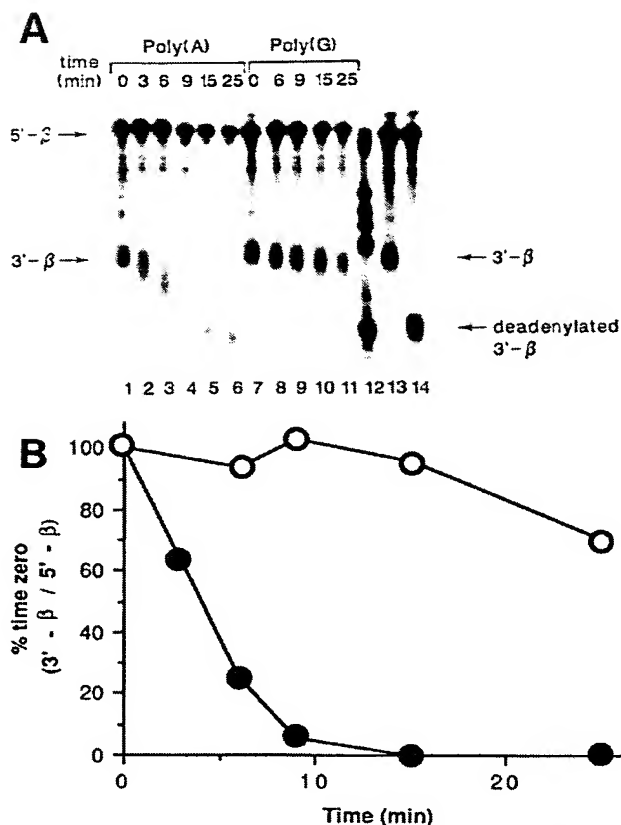


FIG. 9. Accelerated poly(A) shortening and 3'-to-5' degradation in reactions containing competitor poly(A). (A) 32 P-labeled, polyadenylated β -globin mRNA was incubated for the indicated times in reactions containing RSW and 150 ng of either poly(A) or poly(G). RNA was extracted and hybridized with a 25-nucleotide single-stranded deoxynucleotide complementary to a region located 106 to 130 nucleotides 5' of the β -globin poly(A) addition site. RNase H treatment was performed as previously described (11), and the 32 P-RNA fragments were electrophoresed in 8% polyacrylamide-7 M urea gels. 5'- β and 3'- β refer to the two RNase H-generated fragments (Fig. 8). Deadenylated 3'- β fragment was made by annealing the 32 P-mRNA with both the antisense oligonucleotide and oligo(dT) prior to RNase H digestion. Lanes 1 to 11: Reactions incubated for the indicated times (minutes). Lane 12: pBR322 DNA *Hae*III marker; the two fragments migrating at approximately the same position as the deadenylated 3'- β fragment contained 123 and 124 nucleotides; the fragments migrating slightly more slowly than the 3'- β fragment contained 184 and 192 nucleotides. Lane 13: Unincubated reaction. Lane 14: Unincubated reaction; RNA was annealed with both the antisense oligonucleotide and oligo(dT) before RNase H digestion. (B) Bands corresponding to the 5' and 3' fragments were scanned with a soft-laser densitometer to determine relative amounts. The ratios of undegraded fragments were plotted for reactions containing poly(A) (●) or poly(G) (○).

protected the 3' region from nuclease attack. Intermediate-sized mRNA molecules generated during the first 9 min migrated between the undegraded 3' fragment [with its poly(A) tract] and the deadenylated 3' fragment. Since the 5' ends of the 3' fragments were determined by oligonucleotide-directed RNase H cleavage, the intermediate-sized RNAs must have corresponded to molecules undergoing

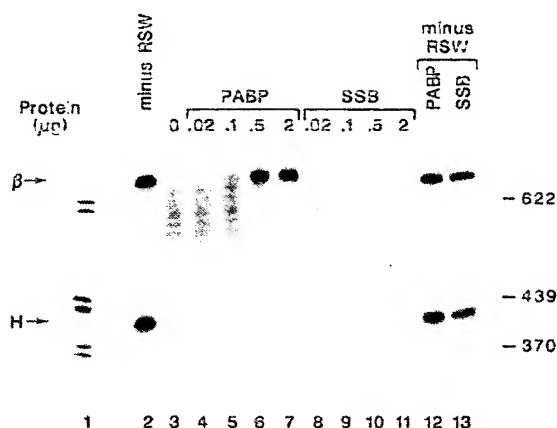


FIG. 10. Lack of effect of *E. coli* SSB on mRNA stability. 32 P-labeled β -globin and H4 histone mRNA substrates were incubated at 37°C for 40 min in reactions containing PABP-depleted RSW. *E. coli* SSB or yeast cytoplasmic PABP was added in the amounts (micrograms per reaction) noted. RNAs were isolated and electrophoresed in 4% polyacrylamide-7 M urea gels. Lane 1: pBR322 DNA *Hae*II marker; fragment sizes in nucleotides are noted on the right. Lane 2: Reaction lacking RSW; undegraded β -globin and H4 histone mRNAs are indicated by β and H, respectively. Lane 3: Complete reaction (with RSW) incubated for 40 min at 37°C; no PABP or SSB was added. Lanes 4 to 11: Reactions with the indicated amounts of PABP or SSB incubated at 37°C for 40 min. Lanes 12 and 13: Controls for nuclease activity in PABP or SSB; reaction mixtures contained 2 μ g of PABP or SSB plus all of the standard reaction components except RSW.

poly(A) shortening. Poly(A) shortening and 3'-to-5' degradation were also observed in reactions with PABP-depleted RSW (data not shown), indicating that the results shown in Fig. 9 were not generated by some artifact related to competitor poly(A).

To determine if any RNA-binding protein would stabilize polyadenylated mRNA, we incubated reactions containing PABP-depleted RSW with either yeast cytoplasmic PABP or *E. coli* SSB. PABP, but not SSB, stabilized the mRNA (Fig. 10). The quantity of SSB in these reactions was sufficient to retard 100% of the mRNA in a gel migration assay (49; P. Bernstein and S. W. Peltz, unpublished observations). Therefore, although SSB could bind to the mRNA, it did not protect it from rapid degradation. Bovine serum albumin also failed to affect mRNA decay (Fig. 3 and data not shown).

To determine if the poly(A)-PABP complex protected other polyadenylated substrates, we tested CAT and SV40 16S late 32 P-mRNAs in vitro with PABP-depleted RSW. Both mRNAs are polyadenylated, and their half-lives are 2 h or more in cells (4, 27). Each was degraded more slowly in the presence than in the absence of PABP (Fig. 11). As estimated by scanning densitometry of the full-length mRNA bands, PABP retarded decay by at least 3- and 10-fold for the SV40 and CAT mRNAs, respectively.

DISCUSSION

These results with an in vitro mRNA decay system support the idea that poly(A) is an important component in determining mRNA stability. It protects some cellular and

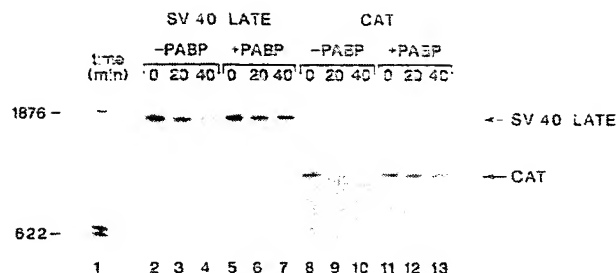


FIG. 11. Polyadenylated SV40 16S late mRNA and CAT mRNA stabilized by PABP. 32 P-labeled, polyadenylated substrates were prepared as described in Materials and Methods and incubated in reactions with PABP-depleted RSW. Where indicated, 2 μ g of yeast PABP was added. After incubation for the indicated times (minutes) at 37°C, RNAs were extracted and analyzed by electrophoresis in 3% polyacrylamide-7 M urea gels. The positions of the undegraded mRNAs are noted on the right. Lane 1: pBR322 *Hae*II DNA marker; fragment sizes in nucleotides are noted on the left. Lanes 2 to 7: SV40 16S late mRNA. Lanes 8 to 13: CAT mRNA.

viral mRNAs from indiscriminate destruction and does so by virtue of its interaction with PABP. For these experiments, we chose to analyze a normally very stable mRNA, β -globin, to evaluate the extent to which the poly(A)-PABP complex affected mRNA stability. The major finding was that, when this mRNA lacks the complex, its turnover rate is drastically altered and becomes similar to that of another mRNA which is normally unstable. In other words, large differences in mRNA turnover rates are nullified when the complex is disrupted. Therefore, the complex probably plays an important role in determining the turnover rates of individual mRNAs. Our results do not suggest that all poly(A)-deficient mRNAs are necessarily very unstable. Mammalian cells contain significant quantities of functional mRNA molecules that, because they do not bind to poly(A) affinity substrates, are thought to be either deficient in or completely lacking poly(A) (22, 31, 32, 66; reviewed in reference 46). Some of these mRNAs seem to have relatively long half-lives, a fact consistent with our finding that different poly(A)-deficient mRNAs are degraded at slightly different rates in vitro (48).

Three in vitro observations suggest that the rate of poly(A) shortening can be a major factor in determining mRNA half-lives. (i) The stability of γ -globin and *c-myc* mRNAs correlates with their poly(A)-shortening rates (11). *c-myc* poly(A) is shortened rapidly, and the mRNA is unstable. γ -Globin poly(A) is shortened slowly, and the mRNA is stable. (ii) Poly(A) removal precedes degradation of the *c-myc* mRNA body (11). This result suggests, but does not prove, that poly(A) must be removed before internal sequences become susceptible to nuclease attack. (iii) A stable mRNA becomes unstable when its poly(A) tract is removed (28, 29, 48; Fig. 7). If the rate of poly(A) shortening determines the nuclease susceptibility of some mRNAs, what accounts for differences in poly(A)-shortening rates between stable and unstable mRNAs? Why should one poly(A) tract be degraded faster than another, when their primary structures are identical, disregarding size differences? Based on our observation that PABP retards poly(A) shortening (Fig. 9), we suggest that any model to account for differential poly(A)-shortening rates should probably invoke disruption of the poly(A)-PABP complex as an essential step. In other words, it might be necessary for PABP to dissociate from the

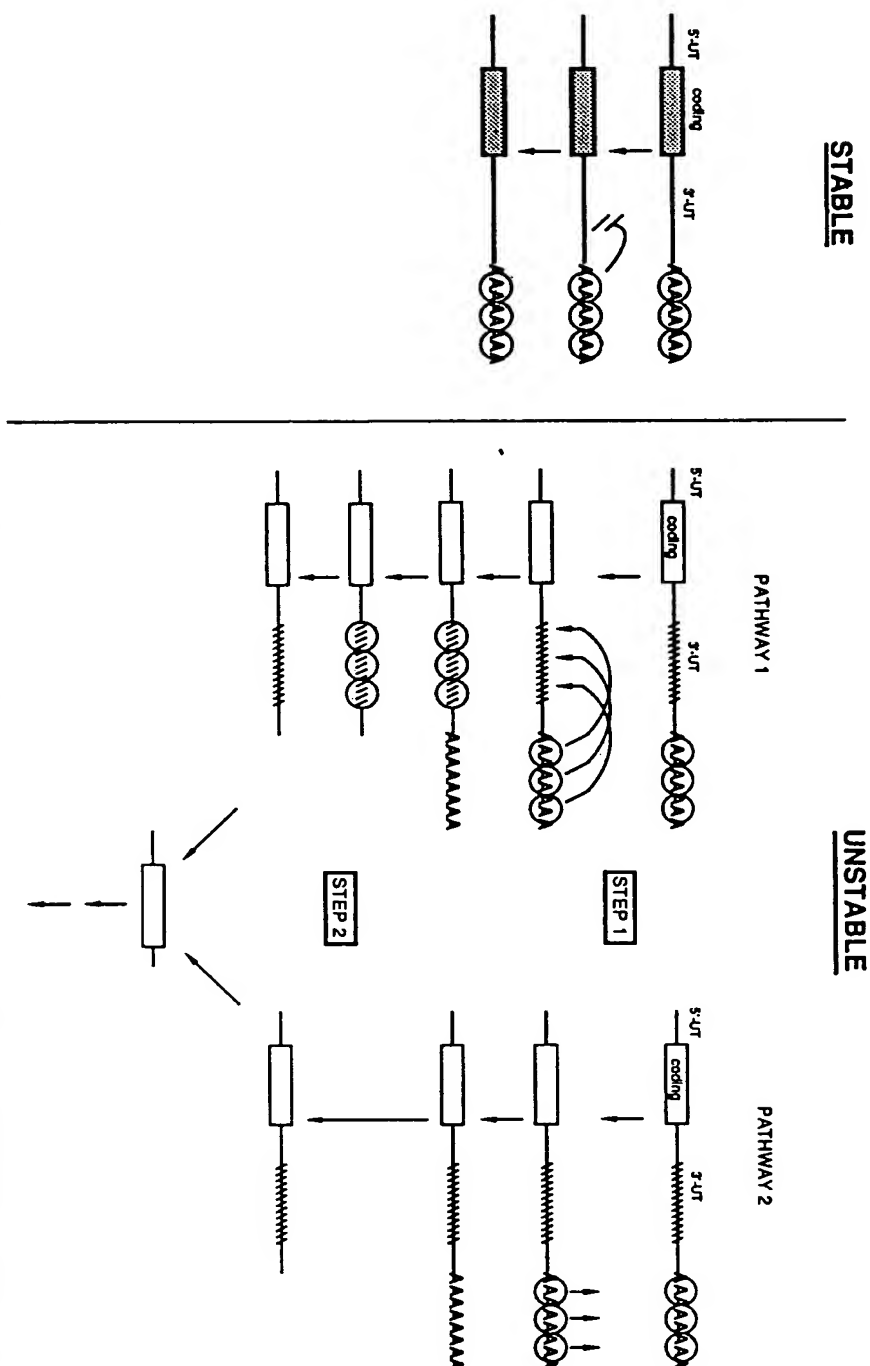


FIG. 12. Model for the role of cis-acting mRNA sequences in determining the stabilities of the poly(A)-PABP complex and the mRNA body. The open circles designate PABP. The hatched lines in the unstable mRNA designate sequences that induce PABP to dissociate from the poly(A) tract, thereby increasing the susceptibility of poly(A) to nucleases. Since the stable mRNA is deficient in dissociation-inducing sequences, the PABP stays bound to its poly(A) tract for a longer time, and the mRNA is therefore more stable. Although the diagram shows the dissociation sequences in the 3'-untranslated region, they could be anywhere in the mRNA. The only restriction on these sequences is that they function in *cis*, not in *trans*. They could function in an indirect fashion, for example, by acting as binding sites for cellular factors that affect the stability of the poly(A)-PABP complex. Two pathways for PABP dissociation are shown. In pathway 1 the PABP migrates from the poly(A) to within the mRNA itself. In pathway 2 it simply dissociates from the poly(A). The ability of PABP to dissociate or to migrate probably results from the presence of four separate RNA-binding sites in a single PABP molecule (2, 23, 58, 59). See Discussion for details.

poly(A) tract before poly(A) shortening (and mRNA degradation) can begin.

One testable model is based on the mobility of PABP, that is, its ability to move from one binding site to another (59, 60; see also references 1, 36, and 65). There are two essential aspects of the model (Fig. 12). (i) PABP remains bound for a longer time to the poly(A) tract of a stable mRNA than to that of an unstable one. (ii) The lifetime of the poly(A)-PABP complex is determined by *cis*-acting sequences in the mRNA body. Unstable mRNAs contain sequences (hatched lines in Fig. 12) that somehow promote the dissociation of PABP from the poly(A) tract, while stable mRNAs lack or are deficient in such sequences. As a result of PABP dissociation (step 1), the unprotected poly(A) becomes nuclease susceptible. If it is attacked by nucleases before another PABP molecule can bind to it, the poly(A) shortens and eventually disappears (step 2), leaving a deadenylated mRNA that is more susceptible than its polyadenylated counterpart to further nuclease attack.

This model is consistent with the *in vitro* data presented here. For example, when PABP dissociation is induced by the presence of excess competitor poly(A), a normally stable mRNA is destabilized (Fig. 1). The model also provides an explanation for how *cis*-acting sequences might affect poly(A)-shortening rates. It thereby links poly(A) metabolism and mRNA turnover with mRNA primary structure, which is the ultimate determinant of mRNA stability. Recent experiments have identified specific sequences which can affect mRNA stability (12, 57, 63, 68), and it would be interesting to know if they do so by affecting the poly(A)-PABP complex. The model shows these sequences in the 3'-untranslated region of the mRNA, but they could just as well be elsewhere. Moreover, the model assumes that there is a limited pool of free PABP in cells. If cells contained a vast excess of PABP, the protein-free poly(A) might bind another PABP molecule so rapidly that poly(A) shortening would rarely occur.

The model shows two possible pathways for PABP dissociation. In the first, PABP migrates away from the poly(A) and reassociates transiently with specific regions in the unstable mRNA. In the second, it dissociates but does not interact further with the mRNA. If reassociation were to occur, it seems unlikely that PABP would bind directly to the mRNA. Although PABP can bind to RNA sequences other than poly(A) (36, 59, 65), a direct (protein-RNA) binding mechanism would require that PABP migrate from a high-affinity to a lower-affinity RNA-binding site. Therefore, it seems more likely that any internal PABP-binding sequences would function indirectly, perhaps as ribonucleoprotein structures in which the protein components act as the inducers of PABP dissociation.

Since some mRNA decay pathways are the same *in vitro* and in whole cells (see above), we suggest that the poly(A)-PABP complex plays an important role in differential mRNA turnover in cells. Some experiments support this suggestion. For example, poly(A) shortening seems to precede degradation of the body of many cellular mRNAs (13, 20, 25, 39, 40, 52, 68, 70, 73). This observation, coupled with the results presented here, indicates that some mRNAs remain intact until their poly(A) tracts are removed. Recent *in vitro* experiments with *c-myc* mRNA support this conclusion (11), as do experiments showing that the stability of growth hormone mRNA in cells is increased when its poly(A) tract is elongated (45). Some oocyte microinjection experiments revealed no correlation between stability and poly(A) length for $\alpha_{2\mu}$ -globulin mRNA (16), and the apparent rate or extent

of poly(A) shortening for actin and tubulin mRNAs in differentiating erythroleukemia cells did not correlate with the rates at which these mRNAs were degraded (34, 42). These results are not incompatible with a poly(A) protection mechanism, because the poly(A)-shortening rate need not be uniform throughout the lifetime of a poly(A) tract. Furthermore, our results and others as well (39, 44, 48) indicate that an mRNA molecule can be protected (stabilized) as long as its poly(A) tract maintains the minimum size (25 to 32 bases) necessary to bind a single PABP molecule.

It seems likely that poly(A) plays more than one role in mRNA metabolism, affecting not only stability but also translational efficiency (46, 47, 62; reviewed in reference 30). Moreover, some mRNAs seem to be just as stable without as with their poly(A) tracts (41, 62, 64). Thus, there are probably multiple determinants of mRNA stability and several degradation pathways as well, depending on the mRNA. The poly(A)-shortening rate might be important for some, but perhaps not all, mRNAs. The stabilities of some mRNAs might be determined by the efficiency with which they are translated, by the structure or intracellular location of the polysome with which they are associated, or by the amount of free end product they produce (14, 15, 24, 43, 49, 67, 71).

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Two Cellular Proteins Bind Specifically to a Purine-Rich Sequence Necessary for the Destabilization Function of a *c-fos* Protein-Coding Region Determinant of mRNA Instability

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The *c-fos* proto-oncogene mRNA is rapidly degraded within minutes after its appearance in the cytoplasm of growth factor-stimulated mammalian fibroblasts. At least two functionally independent sequence elements are responsible for the lability of *c-fos* mRNA. One of these determinants is located within a 0.32-kb sequence present in the protein-coding region. We demonstrate by gel mobility shift experiments and UV cross-linking that at least two protein factors specifically interact with a 56-nucleotide purine-rich sequence located at the 5' end of the 0.32-kb coding region determinant of mRNA instability (CRDI). One protein is predominantly associated with the polysomes, while the other is detected in the post-ribosomal supernatant. Sequence comparison of members of the *fos* gene family revealed that the high purine content of the protein-binding region is conserved through evolution. Deletion of this region from the 0.32-kb CRDI severely impedes its function as an RNA-destabilizing element. Our results suggest that binding of the two proteins to the purine-rich sequence may participate in the rapid mRNA decay mediated by this 0.32-kb *c-fos* CRDI.

The regulation of mRNA stability has recently been identified as a major control mechanism that governs the abundance of cytoplasmic mRNA in eukaryotic cells. Differential mRNA stability can have a fundamental impact upon gene expression in eukaryotic cells and is essential for the maintenance of flexibility in gene expression (for reviews, see references 7, 11, and 31). In general, there are three main elements involved in the process of mRNA degradation: (i) the structural determinants, or *cis*-acting elements of mRNAs that determine mRNA turnover rates; (ii) the enzymes and other auxiliary *trans*-acting factors that modulate the interactions between the enzymes and mRNAs; and (iii) the signals, cellular or extracellular, that trigger or influence mRNA turnover.

Progress made in the past several years toward the understanding of the molecular mechanisms by which selective and differential mRNA degradation is accomplished has focused mainly on the identification and characterization of *cis*-acting elements and the stimuli that modulate or trigger mRNA decay. The *trans*-acting factors involved in mRNA degradation remain largely undefined in comparison. The recent application of gel mobility shift assay and UV cross-linking detected a variety of RNA-protein (RP) interactions between the various *cis*-acting elements that are involved in mRNA stability and the cytoplasmic proteins (2, 6, 8, 19, 23, 24, 27, 40, 43), demonstrating the feasibility of these assays in the identification of *trans*-acting factors that may participate in mRNA degradation.

The *c-fos* proto-oncogene mRNA is one of the least stable mammalian messages (17, 21). The transient nature of its expression in the cytoplasm in response to a wide variety of extracellular stimuli is critical in controlling the transforming potential of the *c-fos* proto-oncogene (13, 22, 26, 35). Previous work showed that the *c-fos* mRNA is targeted for rapid degradation by at least two distinct mRNA degradation

pathways (14, 18, 33, 37, 41). One pathway recognizes a 75-nucleotide (nt) AU-rich instability determinant (ARE) located within the 3' untranslated region (UTR), and the other recognizes destabilizing element(s) present in the protein-coding region (37). Recently, Shyu et al. (36) reported that a 0.32-kb protein-coding region that is 391 to 709 nt downstream of the human *c-fos* translation initiation codon can act as an RNA-destabilizing element. The first two-thirds of this region includes sequences coding for the basic motif and the leucine zipper domain (Fig. 1A). Although the two destabilizing elements share no sequence homology and are located at distinct regions of the *c-fos* transcript, they both appear to direct rapid removal of the poly(A) tail (deadenylation) as a crucial first step that triggers *c-fos* mRNA decay (36). Mutation of the ARE further indicates that this element controls two steps in the mechanism of *c-fos* mRNA degradation: removal of the poly(A) tail, which does not require AUUUA motifs within the ARE, and subsequent decay of the body of the transcript, which appears to be dependent on the intact AUUUA pentanucleotides (36). We recently identified four different U-rich sequence-binding proteins (URBPs) that specifically interact with the *c-fos* ARE (43). The binding sites for the four proteins were all mapped to a 20-nt U-rich sequence located in the 3' half of the *c-fos* ARE, which contains no AUUUA pentanucleotides. We proposed that UBP binding may be involved in the first step, removal of the poly(A) tail, in the *c-fos* ARE-mediated decay pathway.

As an effort to better elucidate the mRNA decay mechanism mediated by the 0.32-kb coding region determinant of mRNA instability (CRDI), we used the gel mobility shift assay and UV cross-linking to identify *trans*-acting factors that may participate in the 0.32-kb CRDI-directed decay through interaction with this region. These experiments revealed two different RNA-binding proteins that form specific RP complexes with a 56-nt purine-rich sequence at the 5' end of the 0.32-kb *c-fos* CRDI. More importantly, deletion of the purine-rich binding sequence from the 0.32-kb CRDI

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significantly impeded its RNA-destabilizing function, demonstrating that the defined purine-rich region is necessary for the 0.32-kb *c-fos* CRDI to act as an RNA destabilizer. Interestingly, the binding activity of one protein factor was detected predominantly in the polysomes, whereas the binding activity of the other was present exclusively in the post-polysomal preparation (S130). The implications of the observed different subcellular distribution of the two protein factors, translation, and mRNA turnover are discussed.

MATERIALS AND METHODS

Cell culture, DNA transfection, and lysate preparation. Culturing, transient transfection, and serum stimulation of mouse NIH 3T3 cells were performed as described previously (37). The preparation of cytoplasmic lysates from NIH 3T3 cells has been described previously (43). The preparation of polysomes and S130 from NIH 3T3 cells was done as described by Brewer and Ross (9).

Plasmid constructs. When necessary, DNA with 5'- or 3'-protruding ends was treated with Klenow fragment or T4 DNA polymerase to make the ends blunt. Standard polymerase chain reaction (PCR) techniques (34) were employed to construct plasmids pCRDI-A, pCRDI-B, pCRDI-C, and pCRDI-E. A human *c-fos* cDNA clone kindly provided by I. Verma served as the template for PCR amplification of the desired regions. The amplified fragments span sequences encoding various segments of the *c-fos* protein-coding region: pCRDI-A, 344 to 712 nt downstream of the *c-fos* AUG codon; pCRDI-B, 344 to 487 nt downstream of the *c-fos* AUG codon; pCRDI-C, 488 to 712 nt downstream of the *c-fos* AUG codon; and pCRDI-E, 391 to 487 nt downstream of the *c-fos* AUG codon. All the PCR-amplified fragments carry a *Bam*HI site at the promoter-distal end. After *Bam*HI digestion, the respective fragments were subcloned between the following two sites in plasmid pT7/T3 α -18 (Bethesda Research Laboratories) to generate the indicated plasmid(s): *Pst*I (blunt ended) and *Bam*HI to generate pCRDI-A, *Hinc*II and *Bam*HI to generate pCRDI-B, and *Sph*I (blunt ended) and *Bam*HI to generate pCRDI-C and pCRDI-E. Plasmid pCRDI-G was generated by deleting the 105-bp *Pst*I fragment from plasmid pCRDI-B. To construct plasmid pT18 F3'C, a 456-bp *Stu*I-*Bgl*II fragment containing the last 432 bp of the carboxy-terminus-coding region plus the first 24 bp of the 3' UTR of the human *c-fos* gene was first excised from plasmid pBFB (37) and was then inserted between the *Pst*I (blunt-ended) and *Bam*HI sites of plasmid pT7/T3 α -18 (Bethesda Research Laboratories). To generate plasmid pT3 β G, a 188-bp *Nco*I (filled-in)-*Bam*HI fragment was excised from plasmid pSVB10 (37) and subcloned between the *Hinc*II and *Bam*HI sites of plasmid pT7/T3 α -19 (Bethesda Research Laboratories).

Plasmids pFBcd66 Δ 1 and pFBcd66 Δ 2 were prepared in several steps as follows. A *Hpa*I site and a *Stu*I site were introduced into the 0.32-kb *c-fos* CRDI region present in plasmid pFBcd66 (36) by site-directed mutagenesis (15) at positions that are 487 (GATACACT \rightarrow GGTTAACT) and 709 nt (GAGGCATG \rightarrow GAGGCCTG) downstream of the *c-fos* AUG codon, respectively (please refer to Fig. 1B for the numbering), to generate plasmid pFBcd66HS. A 488-bp *Bam*HI-*Hpa*I fragment of pFBcd66HS was then excised, and the ends were filled in and religated to generate plasmid pFBcd66 Δ 1. Plasmid pFBcd66 Δ 2 was created by deleting a 336-bp *Hpa*I-*Stu*I fragment from pFBcd66HS.

Preparation of RNA transcripts. Transcription reactions were performed according to the instructions of Promega,

using SP6 or T3 RNA polymerase. Labeled RNA transcripts were produced by including [α - 32 P]UTP (800 Ci/mmol [Amersham or New England Nuclear]) or [α - 32 P]GTP (400 [Amersham] or 800 [New England Nuclear] Ci/mmol) in the reaction mixtures.

The RNA probes were as follows: probe A, a 392-nt sense transcript synthesized from *Stu*I-linearized pCRDI-A; probe B, a 180-nt sense transcript synthesized from *Bam*HI-linearized pCRDI-B; probe C, a 238-nt sense transcript synthesized from *Stu*I-linearized pCRDI-C; probe D, a 130-nt sense transcript synthesized from *Pst*I-linearized (blunt-ended) pCRDI-A; probe E, a 115-nt sense transcript synthesized from *Bam*HI-linearized pCRDI-G; probe F, a 72-nt sense transcript synthesized from *Pst*I-linearized (blunt-ended) pCRDI-E; probe G, a 75-nt sense transcript synthesized from *Bam*HI-linearized pCRDI-G; probe F3'C, a 499-nt sense transcript synthesized from *Eco*RI-linearized pT18F3'C; and *c-fos* ARE RNA, a 109-nt sense transcript synthesized from *Xho*I-linearized pT3ARE (43). A plasmid kindly provided by J. Ross (2) was linearized with *Eco*RI for synthesis of a 182-nt sense *c-myc* RNA.

Analysis of RP interactions. Binding reactions were performed as described previously (43) with minor modifications. Cytoplasmic lysate (7 μ g of protein, with 1 μ g of protein equivalent to $\sim 1.4 \times 10^4$ to 1.7×10^4 cells and 32 P-labeled RNA (1 ng) were incubated at room temperature for 15 min in a buffer containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.6), 3 mM MgCl₂, 40 mM KCl, 2 mM dithiothreitol, 10% glycerol, and 0.5% Nonidet P-40. Heparin (final concentration, 1 mg/ml) and *Escherichia coli* tRNA (final concentration, 200 μ g/ml) were added to reduce nonspecific binding. The volume of each reaction mixture was 10 to 15 μ l. Subsequently, unbound RNA was digested for 20 min by of RNase A (500 μ g/ml) at room temperature. RP complexes were resolved in nondenaturing 6% polyacrylamide gels (20).

For UV cross-linking of RNA and protein, the reaction mixtures were put on ice immediately after RNase A digestion and UV-irradiated for 35 min at 4°C, as described previously (38, 43). The samples were analyzed by electrophoresis on a sodium dodecyl sulfate (SDS)-7.5% polyacrylamide gel under reducing conditions.

Analysis of mRNA decay. Total cytoplasmic RNA was isolated at various times after serum stimulation of transiently transfected NIH 3T3 cells and was analyzed by RNase protection for mRNA decay, as described previously (37). Decay of mRNA was quantitated directly by scanning gels on a Betascope 603 Blot Analyzer (Betagen). Calculation of half-life values has been described previously (37). Human α 1-globin mRNA was detected with a 270-nt RNA probe derived from *Bam*HI-linearized plasmid pSP6 α 133 (10). FBcd66, FBcd66 Δ 1, and FBcd66 Δ 2 mRNAs were detected with a 237-nt RNA probe derived from *Hind*III-linearized plasmid pT3 β G.

RESULTS

Identification of specific RP interactions in the 0.32-kb CRDI of *c-fos* mRNA. As an initial attempt to identify *trans*-acting factors that may participate in the decay pathway mediated by the 0.32-kb CRDI, gel mobility shift assays were performed. Uniformly radiolabeled RNA containing the 0.32-kb sequence plus an extra 47-nt sequence, which is highly conserved in the *fos* gene family (44) and is immediately upstream of the 0.32-kb region (Fig. 1A, probe A), was incubated with cytoplasmic lysate prepared from serum-

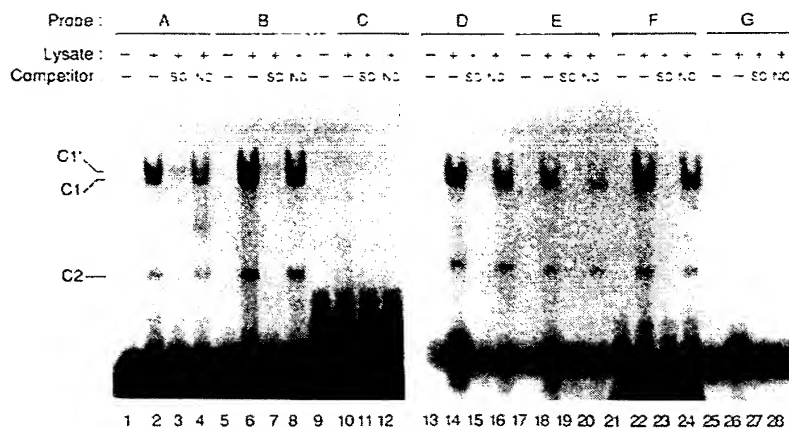


FIG. 3. Mapping of protein-binding region in the 0.32-kb human *c-fos* CRDI. RP binding reactions were carried out as described in the legend to Fig. 2. Gel mobility shift assays of various 32 P-labeled RNA transcripts (1 ng each of probes A, B, C, D, E, F, and G [lanes 1, 5, 9, 13, 17, 21, and 25, respectively]) were performed to test for their ability to form band-shifted complexes with cytoplasmic lysate (7 μ g of protein) in the absence (lanes 2, 6, 10, 14, 18, 22, and 26) or presence of 500-fold molar excess of either unlabeled probe A RNA (specific competitor [SC]) (lanes 3, 7, 11, 15, 19, 23, and 27) or unlabeled F3'C RNA (nonspecific competitor [NC]) (lanes 4, 8, 12, 16, 20, 24, and 28) as indicated. C1', C1, and C2 denote the positions of the three band-shifted complexes.

containing just a 56-nt region from the 5' portion of the 0.32-kb CRDI (Fig. 1B, boldface type). These results show that the RP interaction occurs specifically and exclusively at the 5' 56-nt segment of the 0.32-kb *c-fos* CRDI. Interestingly, the 56-nt protein-binding region is extremely rich in adenylate and guanylate residues, i.e., approximately 77% are purine residues.

To determine directly the identities of the protected RNA fragments that interact with each of the three RP complexes, the three band-shifted complexes were excised from a native gel, and the protected [32 P]GTP-labeled RNA fragments from each gel slice were analyzed by further electrophoresis on a 16% urea gel (Fig. 4). RNA fragments generated from the complete RNase A digestion of 32 P-labeled probe E RNA were used as size markers. Because probe E RNA contains very few pyrimidine residues (Fig. 1B) and RNase A cleaves after pyrimidine residues, unique purine-rich fragments with



FIG. 4. Identification of the RNase A-resistant fragments in complexes C1', C1, and C2. RP binding reactions using 32 P-labeled E RNA as the substrate were carried out as described in the legend to Fig. 2. 32 P-labeled RNase A-resistant RNA fragments present in each of the RP complexes were analyzed by further electrophoresis of the excised gel slices containing individual band-shifted complexes on a denaturing 16% polyacrylamide gel (lanes 2 to 4) and compared with the sizes of RNase A-digested 32 P-labeled E RNA (lane 1). The positions (in nucleotides) of RNA size markers are indicated to the left of the gel.

sizes of 24, 14, and 12 nt would be generated along with several smaller fragments ranging from 2 to 5 nt after RNase A digestion of probe E RNA. Note that the 24- and 14-nt fragments are derived from the 56-nt protein-binding region defined by mapping experiments (Fig. 1B and Fig. 3). As shown in Fig. 4, while the resulting RNA fragments from complexes C1' and C1 show an identical subset of fragments of 24 and 14 nt, the protected RNA fragments isolated from complex C2 comigrates with just the unique 12-nt fragment. These results are completely consistent with the mapping data (Fig. 1A and Fig. 3) and also show that formation of all three complexes involves RNA fragments generated from the 56-nt region, i.e., 24- and/or 14-nt fragments. As shown in Fig. 1B, both fragments are extremely purine-rich and contain a continuous purine sequence followed by a single uridylylate residue that is left behind after RNase A digestion. Because the unique 24- and 14-nt fragments are separated by only 2 nt, we have further narrowed down the protein-binding site within the 0.32-kb CRDI to a 40-nt region.

Characterization of the 0.32-kb *c-fos* CRDI-binding proteins by UV cross-linking. To estimate the apparent molecular weight of the 0.32-kb CRDI-binding proteins and characterize the protein composition of each complex, UV-induced cross-linking of RNA and protein was performed (43). The binding mixture was analyzed by electrophoresis on a SDS-7.5% polyacrylamide gel following UV irradiation. A 64-kDa band was detected by UV cross-linking (Fig. 5, lane 2). The 64-kDa band did not occur when 500-fold molar excess of unlabeled homologous probe E RNA (Fig. 5, lane 3) was included in the binding reaction, whereas 500-fold molar excess of an unlabeled F3'C RNA (Fig. 5, lane 4) had no effect at all. As shown above (Fig. 2 and Fig. 3), three distinct RP complexes are detected by gel mobility shift assay. Therefore, observation of a single band by UV cross-linking and SDS-polyacrylamide gel electrophoresis (PAGE) raises the question whether formation of the three complexes might involve the same cellular protein factor. To address this issue, protein composition of the three complexes was further determined as follows. Complexes C1',

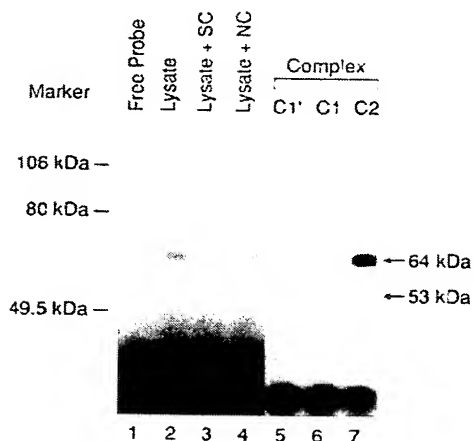


FIG. 5. Identification of polypeptides interacting with the purine-rich RNA substrate by UV cross-linking. RP binding reactions using 32 P-labeled E RNA as the substrate were carried out in the absence (lanes 2 and 5 to 7) or presence of 500-fold molar excess of either unlabeled probe E RNA (specific competitor [SC]) (lane 3) or unlabeled F3'C RNA (nonspecific competitor [NC]) (lane 4) as described in the legend to Fig. 2. Following incubation with RNase A, 32 P-labeled RP complexes were UV cross-linked as described in Materials and Methods. Lane 1, free probe. Lanes 2 to 4, the cross-linked products were directly resolved by SDS-7.5% PAGE. Lanes 5 and 6, the cross-linked products were first subjected to electrophoresis on a nondenaturing low-ionic-strength 6% polyacrylamide gel. Individual band-shifted complexes were excised from the gel and boiled in $1\times$ protein sample buffer for 2 min prior to SDS-7.5% PAGE. Prestained molecular mass standards (Bio-Rad) were used as markers.

C1, and C2 were separately excised from the native gel following electrophoresis of UV-irradiated binding mixture. The individually excised complexes were subjected to further electrophoresis on a denaturing SDS gel. While complex

C2 was resolved into a single 64-kDa band, both complex C1' and complex C1 gave rise to a faint band with an apparent molecular mass of 53 kDa (Fig. 5, lanes 5 and 6). Therefore, formation of the three complexes involves at least two different proteins. It is likely that lack of readily detectable band(s) from complexes C1' and C1 on the SDS gel, as shown in Fig. 5, lanes 2 and 4, is due to particularly low efficiency of UV-induced transfer of 32 P label to the cellular protein(s) (32, 43). Taken together, the results of these experiments and the experiment of Fig. 4 show that complexes C1' and C1 have the same molecular mass and protect an identical subset of RNA fragments from RNase A digestion. We conclude that the formation of complexes C1' and C1 may involve one protein factor (protein C1) which is different from that of complex C2 (protein C2).

Specificity of RNA-binding proteins and their subcellular distribution. To further explore RNA-binding specificity, various ribohomopolymers were tested for their ability to compete with the 32 P-labeled *c-fos* E probe for binding to the protein factors in a gel mobility shift assay. As shown in Fig. 6A, both unlabeled poly(A) and poly(G) were able to compete for binding, whereas poly(U) and poly(C) showed no competition at all, even at 516 ng. We also tested two other RNAs for their ability to compete with the probe E for protein interaction. They both encode RNA elements involved in message stability and have been shown to interact with distinct cellular proteins by UV cross-linking (2, 43). One is a 75-nt AU-rich element (ARE) in the 3' UTR of the *c-fos* mRNA that is known to function as a destabilizing element (37). The other is a 182-nt RNA encoding a *c-myc* coding region stability determinant (2). Purines make up ~58% of this latter sequence. Neither RNA, when used as a competitor, has an effect on the formation of complexes in the competition experiment (Fig. 6B), indicating that the protein factors involved in the formation of RP complexes with the 0.32-kb *c-fos* CRDI are different from the protein factors interacting with these two RNAs.

The purine-rich, especially adenylate-rich, nature of the 40-nt RNA-binding sequence (Fig. 1B) prompted us to test

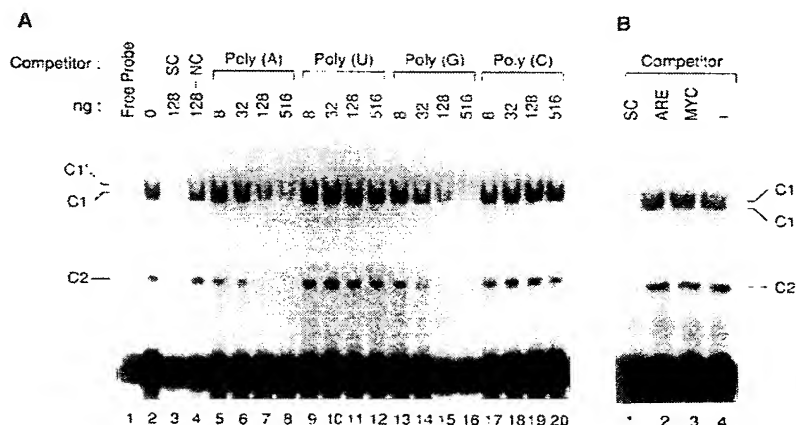


FIG. 6. Specificity of formation of the C1', C1, and C2 complexes. RP binding reactions were carried out as described in the legend to Fig. 2. (A) Lane 1, free 32 P-labeled E RNA. Lanes 2 to 20, RNA-protein binding reactions using 32 P-labeled E RNA as the substrate were performed in the absence (lane 2) or presence of unlabeled E RNA (specific competitor [SC]) (lane 3), F3'C RNA (nonspecific competitor [NC]) (lane 4), or homoribonucleotide polymers (lanes 5 to 20) as indicated. (B) RP binding reactions using 32 P-labeled E RNA as the substrate were performed in the absence (lane 4) or presence of 500-fold molar excess of unlabeled E RNA (lane 1), *c-fos* ARE RNA (lane 2) or *c-myc* coding determinant RNA (lane 3) as indicated. C1', C1, and C2 to the sides of the gels denote the positions of the three band-shifted complexes.

whether the protein factors interacting with it might be poly(A)-binding protein (PABP). Previously Bernstein et al. (3) showed that *Saccharomyces cerevisiae* PABP synthesized in bacteria associates with the poly(A) tail and affects mammalian mRNA stability in an *in vitro* decay system, suggesting that yeast PABP is functionally equivalent to the mammalian PABP. Therefore, we carried out gel mobility shift assays using increasing amounts of purified yeast PABP in the presence or absence of NIH 3T3 cell lysate (data not shown). A faint shifted band in a position different from those of the three complexes was observed when 1.25 μ g of yeast PABP alone was mixed with 32 P-labeled probe E RNA. This result indicates that yeast PABP binds very weakly to the probe E RNA and forms, if any, a different RP complex. Moreover, no competition was detected between the purine-rich sequence-binding proteins and yeast PABP. There is no diminution of formation of the three complexes even when 1.25 μ g of yeast PABP was added to the binding mixture. Therefore, we conclude that the protein factors involved in formation of the three RP complexes are different from PABP.

As an attempt to gain further insight into the mechanism by which binding of the protein factors might lead to degradation of the *c-fos* mRNA, we determined the subcellular distribution of the binding activities of these proteins. Post-ribosomal supernatant (S130) and polysomes prepared from NIH 3T3 cells were tested for their ability to support formation of the RP complexes in a gel mobility shift assay. Interestingly, the binding activity of the C1 protein, as judged by the formation of complexes C1' and C1, was found exclusively in S130 (Fig. 7A). In contrast, binding activity of the C2 protein, as judged by the specific formation of complex C2, was detected mainly in the polysome preparation (Fig. 7), indicating that the C2 protein is associated with polysomes.

The protein-binding sites are necessary but not sufficient for the 0.32-kb *c-fos* CRDI to function as an RNA-destabilizing element. While the detailed structural features that are required for the 0.32-kb *c-fos* CRDI to function as an RNA-destabilizing element *in vivo* are not known, identification of the 56-nt RP interaction region within it may suggest a critical role of the binding sites in determining its destabilizing function. To test this hypothesis, we have chosen to make an in-frame deletion construct from plasmid pFBcd66, which carries the 0.32-kb *c-fos* CRDI inserted in-frame into the coding region of a β -globin gene derivative (Fig. 8A) (36). The lability of the corresponding FBcd66 hybrid message was previously shown to be due to the insertion of the 0.32-kb *c-fos* CRDI by Shyu et al. (36). The deletion, designated FBcd66 Δ 1 (Fig. 8A), removed the first 87-nt segment of the CRDI from FBcd66 that includes the protein-binding region defined by gel mobility shift assay (Fig. 8A). A transient-induction assay (37) was used to determine the effect of the deletion on stability of the FBcd66 mRNA in mouse fibroblasts after growth factor stimulation. Plasmid DNA carrying the FBcd66 gene or its deletion derivative under control of the *c-fos* promoter was introduced along with plasmid pSV α 1 (37), which encodes a stable message and serves as an internal control for transient transfection, into mouse NIH 3T3 cells by transient transfection. After serum starvation for 25 to 30 h, transcription was induced transiently by stimulation with calf serum, and total cytoplasmic RNA was isolated at various time intervals. The relative concentrations of these mRNAs were then determined by RNase protection analyses (Fig. 8B).

As reported by Shyu et al. (36), the FBcd66 transcript

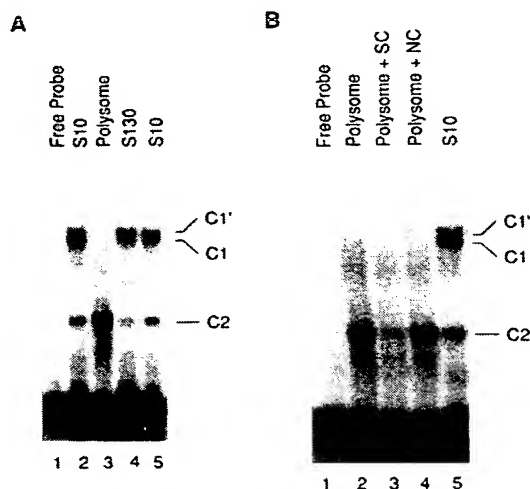


FIG. 7. Subcellular distribution of the binding activity of the two RNA-binding proteins. The post-ribosomal supernatant (S130) and polysomes were prepared from crude NIH 3T3 cell lysate (S10) by the method of Brewer and Ross (9). (A) RP binding reactions were carried out with 32 P-labeled probe E RNA (lane 1) in the presence of S10 (7 μ g of protein; lanes 2 and 5), polysomes (0.4 A_{260} units; lane 3), or of S130 (0.3 A_{260} units; lane 4). (B) Polysome preparation supports specific formation of the C2 complex. The 32 P-labeled E RNA (lane 1) was incubated with the polysomes alone (lane 2) or in the presence of 500-fold molar excess of unlabeled probe E RNA (specific competitor [SC]) (lane 3) or unlabeled F3' C RNA (nonspecific competitor [NC]) (lane 4) as indicated. Migration of the three complexes formed between the 32 P-labeled E RNA and crude cytoplasmic lysate (S10; 7 μ g of protein) are used as markers (lane 5). C1', C1, and C2 to the right of the gels denote the positions of the three band-shifted complexes.

decay rapidly, with a half-life of ~ 50 min (Fig. 8B, lanes 1 to 4). In contrast, the mutant mRNA message with the deletion is very stable (Fig. 8B, lanes 5 to 8). The cytoplasmic half-life of the mutant transcript is ~ 420 min, a value approximately ninefold-more stable than that of the FBcd66 message, indicating that the protein-binding sequence is necessary for the 0.32-kb *c-fos* CRDI to confer a high degree of instability upon the otherwise long-lived β -globin mRNA. The data suggest that binding of these protein factors to the purine-rich sequence may participate in the 0.32-kb CRDI-mediated mRNA decay.

To learn whether the protein-binding region alone is sufficient to function as an RNA-destabilizing element, a reciprocal in-frame deletion to the FBcd66 Δ 1 deletion designated FBcd66 Δ 2 was generated by deleting the *c-fos* CRDI sequence that is immediately downstream from the first 87-nt region from the FBcd66 mRNA (Fig. 8A). This manipulation left intact the 87-nt region that has been shown to be necessary for the destabilization function of the *c-fos* CRDI in the FBcd66 mRNA. The half-life for the FBcd66 Δ 2 message was determined to be ~ 267 min (Fig. 8B, lanes 9 to 12). The results show that the protein-binding region alone has no apparent or very significant destabilizing effect on the stable β -globin mRNA. Taken together, the data show that the protein-binding sequence within the 0.32-kb CRDI is necessary for its destabilization function but the sequence alone is insufficient to act as an RNA-destabilizing element.

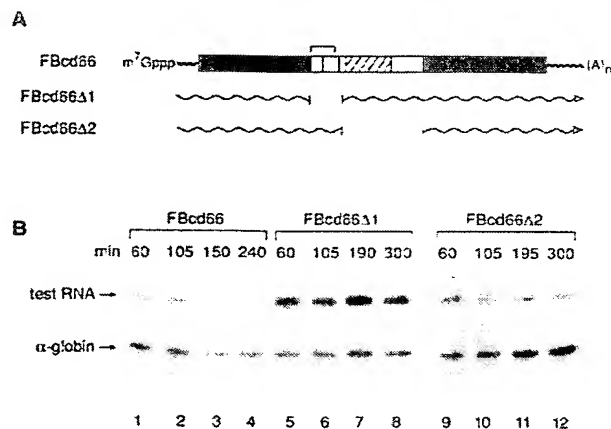


FIG. 8. Schematic representation and decay of FBcd66, FBcd66Δ1, and FBcd66Δ2 mRNA. (A) Diagrams of FBcd66, FBcd66Δ1, and FBcd66Δ2 mRNA. The open rectangle represents the 0.32-kb *c-fos* CRDI. The regions encoding the *c-fos* protein basic motif (hatched box) and leucine zipper domain (stippled box) are indicated. Solid rectangles represent the β -globin-coding region segments. The thick wavy lines attached to the solid rectangles represent β -globin UTRs. (A)_n indicates the 3' poly(A) tail. The 56-nt protein-binding region is indicated by the bracket on the top. The wavy lines and arrows underneath the diagram of FBcd66 mRNA denote the portions that are carried by the FBcd66Δ1 or FBcd66Δ2 chimeric mRNA. (B) Decay of FBcd66, FBcd66Δ1, and FBcd66Δ2 mRNA. NIH 3T3 cells were transiently cotransfected with pSVa1 and one of the following plasmids: pFBcd66, pFBcd66Δ1, or pFBcd66Δ2. Total cytoplasmic RNA was isolated at various time intervals after serum stimulation and analyzed by RNase protection and gel electrophoresis on denaturing 6% polyacrylamide gels. Test RNA is the pT3βG-derived ³²P-labeled probe fragment protected by FBcd66 (lanes 1 to 4), FBcd66Δ1 (lanes 5 to 8), or FBcd66Δ2 (lanes 9 to 12) chimeric mRNA; α -globin is the pSP6a133-derived ³²P-labeled probe fragment protected by human α -globin mRNA. The times given over the lanes correspond to minutes after serum stimulation.

DISCUSSION

We report here the identification of two novel cellular proteins that interact with a 0.32-kb *c-fos* CRDI to form specific RP complexes that may participate in the 0.32-kb CRDI-directed mRNA decay pathway. Using a variety of ³²P-labeled RNA transcripts corresponding to various portions of the 0.32-kb *c-fos* CRDI in gel mobility shift assays, we were able to narrow down the protein-binding sites to the same 56-nt region at the 5' end of the 0.32-kb *c-fos* CRDI (Fig. 1 and 3). Subsequent isolation of the unique 24- and 14-nt fragments from the RP complexes that were proven to be derived from the 56-nt region is completely consistent with the mapping data (Fig. 1 and 4). The importance of the protein-binding region in determining the destabilization function of the 0.32-kb CRDI was further demonstrated by our deletion analysis (Fig. 8). Our data indicate that the protein-binding sequence is necessary for the destabilization function of the 0.32-kb CRDI. Therefore, the participation of the C1 and C2 proteins in decay directed by the 0.32-kb *c-fos* CRDI may be inferred.

Since the 14-nt fragment is just 2 nt downstream of the 24-nt fragment, it is possible that the RNA portion associated with protein C1 actually consists of both the 24- and 14-nt sequence fragments as a continuous 40-nt sequence

which interacts only loosely with the protein so that RNase A can still attack the pyrimidine residues that link the two fragments together to generate the 24- and 14-nt fragments. Therefore, the 40-nt sequence may represent the binding site for protein C1, within which resides the 24-nt binding sequence for protein C2. As shown in Fig. 4, lanes 2 and 3, the intensity of the 14-nt fragment is stronger than that of the 24-nt fragment, suggesting that the 14-nt fragment alone might be able to interact with protein C1. Thus, alternative possibilities are that there is a somewhat less stringent requirement of sequence specificity and that there is a smaller RNA-binding sequence requirement for the C1 protein to form the C1' and C1 complexes than that for the C2 protein to form the C2 complex.

Remarkably, the protein-binding region of the 0.32-kb CRDI is extremely rich in purine residues, indicating the importance of purines nature in determining the specificity of the RP interaction. The finding that both poly(A) and poly(G) but not poly(U) and poly(C) can compete with the probe for binding also supports this notion (Fig. 6A). However, the RP interaction may not be merely dependent on the purine-rich nature of the sequence. Since each molecule of a 115-nt probe E RNA contains only one 24-nt RNA-binding domain (compared with five RNA-binding domains in a ribohomopolymer with a similar length), if purine richness were the only requirement, both poly(G) and poly(A) would represent a fivefold molar excess over probe E RNA in terms of the protein-binding site when the same amount of RNA was used. However, with a comparable molar excess, both poly(A) and poly(G) appear to be somewhat less effective in competing with probe E for binding to the cellular proteins (compare lanes 2 to 6 in Fig. 2A with lanes 4 to 8 and 13 to 16 in Fig. 6A). Thus, this observation indicates some degree of sequence specificity in the interaction between the proteins and the 0.32-kb CRDI RNA.

As an initial effort to characterize the nature of the purine richness and to identify the potential sequence motif(s) that may contribute to specify the RP interaction, the 40-nt protein-binding region of the human *c-fos* RNA was compared with the corresponding regions of other members of the *fos* gene family (Fig. 9), including regions from the mouse *c-fos* (4), chicken *v-fos* (29), mouse *fosB* (44), human Fos-related antigen 1 (*fra-1* [25]), rat *fra-1* (12), chicken Fos-related antigen 2 (*fra-2* [28]), and human *fra-2* (25). Interestingly, although there is only ~48% RNA sequence homology among the eight genes and there are many sequence changes among these genes, the purine richness of this 40-nt region appears to be maintained through evolution. More than 75% of each gene consists of purine residues. Moreover, when the purine contents of the sequences corresponding to the 24-nt RNA fragment of human *c-fos* are compared, the feature of exclusive purine richness is even more significant (Fig. 9). Therefore, this observation might suggest that purine richness plays a critical role in determine the specificity of the RP interaction, which is highly conserved among these mRNAs of the *fos* gene family. However, other than the conservation of extraordinary high purine content among these sequences, we do not find any apparent sequence motifs. The relative percentage of adenylate and guanilate residues of the 40-nt region also varies to a significant extent among members of the *fos* gene family. It will be of great interest to carry out additional studies to define the nature and structural features of the 40-nt region that are necessary to invoke the specific RP interaction.

Although it seems unlikely that the RP interaction is dependent solely on the overall purine richness, the purine

		% of Purine content	
		40-nt frag	24-nt frag
human c-fos	<u>AGAAGAAGAAGAGAAAAGGAGAAUCCGAAGGGAAAGGAAU</u>	90	96
mouse c-fos	U-----G-----C-----	87	88
chicken v-fos	G--G--G--G--A--G-----G-----CC---G-----C	85	96
mouse fos B	-----A--GC-A--GG-U--C--A--GC-----C	83	92
human fra-1	G--G-----G---CGCC-CC--G-AA-GC-C--GC-----C	75	79
rat fra-1	G--G--G-----CGCC-C--GG-GA--C-C--GC-----C	75	79
chicken fra-2	U--G-----GC-A--G-----G--A--G-----C	85	92
human fra-2	U-----G--G-----GC-UC-C-----GGA-AGG-AC--G	78	75

FIG. 9. Sequence comparison of the purine-rich region of members of the *fos* gene family. The sequence comparison is shown to the left of the figure. The 40-nt RNA sequence of human *c-fos* mRNA that carries the protein-binding sites is shown. The underlined sequences are the unique 24- and 14-nt RNase A-resistant RNA fragments that are involved in the formation of the three band-shifted complexes. Base differences between the human *c-fos* and other members of the gene family are indicated below the human *c-fos* sequence at the corresponding position. Percentages of purine content of the 40- and 24-nt fragment (frag) sequences from members of the *fos* gene family are listed to the right of the figure.

richness, especially adenylate richness, raises the question whether the protein factors we identified may be PABP. Several lines of evidence indicate that proteins involved in the formation of the 64- and 53-kDa bands are different from the cytoplasmic PABP. First, our experiment using yeast cytoplasmic PABP showed that it interacts only weakly with the 0.32-kb RNA to form a different band-shifted complex (data not shown). Second, our competition experiments showed that the addition of increasing amounts of the yeast PABP to the binding mixture has no effect at all on the formation of the three specific RP complexes (data not shown), indicating that yeast PABP could not compete with the two protein factors for binding to the 0.32-kb CRDI. Third, the purified mammalian cytoplasmic PABP has an apparent molecular mass of 72 kDa on SDS-PAGE (1, 5), which is greater than those of the C1 and C2 proteins. Moreover, we were barely able to UV cross-link ³²P-labeled probe E RNA to yeast PABP, even when 1.25 µg of PABP was used, and the RNA-PABP complex has an apparent molecular mass of 80 kDa on a SDS gel, which is significantly larger than the two proteins (data not shown). Taken together, these observations indicate that the two protein factors identified in this study are not PABP. It is, however, noteworthy that the modest but specific affinity of the two proteins to the poly(A) sequence might suggest that they are involved in the process of the 0.32-kb CRDI-mediated decay pathway by facilitating removal of poly(A) tail. How this might be achieved is not known. One might speculate that this might be accomplished by destabilizing the poly(A)-PABP complexes through the specific interaction between the RP complex and the PABP-poly(A) complex so that the poly(A) tail is exposed to exonuclease attack. Alternatively, the RP interaction may lead to subsequent recruitment or activation of a poly(A)-specific nuclease.

How might the initial recognition or interaction of the protein factors and the 0.32-kb *c-fos* CRDI occur in vivo? Our finding that the binding activity of the C2 protein is predominantly associated with the polyribosomes is quite interesting and may suggest that in vivo the polysome-associated protein C2 may be brought to the proximity of the *cis*-acting elements by translating ribosomes to facilitate the RP interaction. This would then require that the 0.32-kb *c-fos* CRDI-directed mRNA decay be coupled to protein translation. This notion is consistent with the previous finding that blocking translation of the *c-fos* coding region by a translation elongation inhibitor, cycloheximide, prevents

c-fos mRNA from rapid degradation (16). In addition, this notion is also consistent with several studies describing the coupling of mRNA decay to protein translation. For example, Yen et al. (42) proposed that tubulin mRNA degradation may use a nuclease that is bound to or activated by translating ribosomes. Recently, Bernstein et al. (2) reported that they identified a polysome-associated protein that is capable of binding to a coding region stability determinant of *c-myc* mRNA and in turn stabilize *c-myc* mRNA decay in their in vitro system. Although not a coding region determinant, the stem-loop structure at the very 3' end of histone mRNA that is responsible for cell cycle- and translation-dependent destabilization of histone mRNA has also been shown to bind a polysome-associated protein factor (30). The RNA-polysome-associated protein interaction has been proposed to play a direct role in mediating histone mRNA decay (30). Therefore, the coupling of mRNA turnover to translation may be accomplished via polysome-associated protein factors (nuclease or nonnuclease). Taken together, it is possible that translation of the *c-fos* message might bring the C2 protein and the 24-nt *cis*-acting element in close proximity that allows the interaction to occur in a translation-dependent and message-specific manner. Given the fact that the C2 protein has some affinity to the poly(A) sequence, the RP interaction might in turn result in subsequent mRNA decay by facilitating the removal of poly(A) tail. However, since the nature of the C2 protein, its relationship to translation, and how the RP interaction may cause or activate mRNA decay remain to be further defined, it is still likely that the C2 protein may participate in steps that follow poly(A) tail removal.

What might be the role of the C1 protein present in the post-polysome preparation (S130)? How might it be involved in the 0.32-kb CRDI-mediated decay pathway? Does it compete with the C2 protein for binding to the 40-nt purine-rich sequence or does it actually bind along with the C2 protein to form a larger RP complex? While the nature of this protein and its precise function await further investigation, identification and isolation of a cytosolic protein from S130 that interacts with the *c-myc* AU-rich element in the 3' UTR and results in subsequent rapid decay of *c-myc* mRNA in an in vitro system by Brewer (8) may suggest an analogous role of the protein C1 in targeting *c-fos* for rapid mRNA decay. An in vitro-reconstituted system will help to address these issues.

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